

Drainage versus defense

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Drainage versus defense:
The management of vascular leakage in
cardiovascular diseases

Rinrada Kietadisorn

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Drainage versus defense:
The management of vascular leakage in
cardiovascular diseases

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Chapter 1

General introduction

Current status of cardiovascular diseases

Cardiovascular disease (CVD), such as heart disease (ischemic heart disease, IHD; coronary heart disease, CHD) and cerebrovascular disease (mainly ischemic stroke) collectively account for 45% and 31% of all deaths in Europe ¹ and worldwide ², respectively, and are the two most common single causes of death. Major CVD risk factors fall into two groups: (I) behavioral risk factors (diet, low physical activity, smoking, alcohol use), and (II) medical risk factors (high systolic blood pressure, high total cholesterol, high fasting plasma glucose, and high body mass index (BMI))¹.

Atherosclerosis, a chronic inflammatory disease of large- to medium-sized arteries, is the main underlying cause of CVD ³. Failure of lymphatics in the mediation of drainage of interstitial fluids, the inflammatory response and in clearance of cholesterol from the arterial intima, may contribute to atherosclerosis ⁴. In addition, the hallmark of endothelial dysfunction is the impaired nitric oxide (NO) bioavailability ⁵, leading to increased expression of several inflammatory mediators and adhesion molecules; these processes contribute to increased plaque vulnerability ⁶. The search for novel and improved treatments for atherosclerosis-related CVD remain one of the major challenges in the field of cardiology. To this end we need a better understanding of the relevant pathomechanisms. Importantly, the abnormal development of microvessels leads to their immaturity, fragility and leakage, and is associated with CVD ⁷. This introduction will present a short overview on the role of microvessels and lymphatics, as well as the regulation of their formation in CVD.

Atherosclerosis was first identified as a pathology of cholesterol storage, leading to artery obstruction, however, it is now additionally recognized as a lipid-driven chronic inflammatory disease ^{8, 9}. Atherosclerotic plaques are composed of cholesterol, lipids and cellular debris, deposited in the artery walls, resulting in their narrowing. Usually, atherosclerotic lesions affect large and medium-sized elastic and muscular arteries and can lead to ischemia of the heart, brain, or extremities, resulting in infarction ¹⁰. Thus, major manifestations of atherosclerosis include IHD, ischemic stroke, and peripheral arterial disease (PAD) ¹¹. Unlike IHD and stroke, PAD is rarely lethal and is a less prevalent complication of atherosclerosis ^{11, 12}. Additionally, stenotic coronary arteries with stable atherosclerosis may cause stable angina, but this is rarely fatal. However, plaques that rupture play a major role in the pathogenesis of life-threatening acute coronary syndromes (ACS) and its manifestations include unstable angina, acute myocardial infarction (MI) and heart failure (HF) ¹³.

Plaque rupture leads to thrombus formation, which is considered the main cause of acute cardiovascular events ^{14, 15}. It has been suggested that plaque rupture occurs mainly in the weakest spots (i.e. the cap margin or shoulder region ¹⁶) of

lesions that contain a thin and inflamed fibrous cap covering the large necrotic core (thin-cap fibroatheromas (TFCA) or vulnerable plaques¹⁶). Rupture-prone caps are infiltrated by macrophage foam cells, and contain few smooth muscle cells (SMC), which are indeed absent at the actual site of rupture¹⁶. Notably, plaque macrophages exhibit impaired migratory capacity, which contributes to their failure to resolve inflammation within the plaque, and enhances lesion progression¹⁷. Inflammatory cells such as macrophages and T-cells are thought to be the main drivers of plaque rupture, releasing proteolytic metalloproteinases that inhibit collagen synthesis; causing the weakening of the cap and reducing plaque mechanical stability^{18,19}. The continued inflammatory response ultimately leads to the destabilization of atherosclerotic plaques via the action of proinflammatory cytokines such as interferon gamma (IFNG/ γ), tumor necrosis factor alpha (TNFA/ α) and interleukin 1 beta (IL1B/ β)²⁰. Moreover, dying macrophages release their lipid contents, along with tissue factor, leading to the formation of a pro-thrombotic necrotic core, a key component of unstable plaques which contributes to their rupture¹⁷. This process is even augmented, due to the reduced capacity of plaque macrophages to perform efferocytosis, the process of apoptotic cell internalization and degradation by phagocytic cells (e.g. macrophages). As a result, non-engulfed apoptotic cells eventually become leaky, resulting in secondary necrosis, which contributes to the formation and expansion of the necrotic core, and in turn, increases plaque vulnerability to rupture²¹. The risk of plaque rupture is also associated with angiogenesis and intraplaque haemorrhage (IPH)¹⁴. Indeed, plaque angiogenesis plays an important role in the pathogenesis of atherosclerosis. Plaque vasa vasorum (VV) exhibit abnormal morphology, have aberrant endothelial cell junctions and show increased leukocyte adherence; this immature microvascular network may contribute to plaque progression and vulnerability. The disruption and leakiness of incompetent VV is directly involved in leukocyte and erythrocyte extravasation and therefore in IPH^{7, 22, 23}. Importantly, IPH plays a causal role in promoting the expansion of the necrotic core and enhancing plaque progression via deposition of free cholesterol derived from red blood cells²². IPH can elicit oxidative stress responses through the release of Fe^{++} from entrapped hemoglobin after red blood cell lysis. In parallel, hemorrhages stimulate neutrophil recruitment. These neutrophils can then release pro-oxidant enzymes into the plaque, causing endothelial cytotoxicity, subsequent acceleration of disease progression, and ultimately plaque rupture²³.

Interestingly, recent observations suggest that multiple TCFA coexist in patients, and that thrombotic rupture of TFCA is a rare event, especially in low risk subjects. This subsequently challenges the concept of the vulnerable plaque^{24, 25}. It may also imply that rupture is not solely dependent on plaque morphology; other local mechanical factors are probably involved. In this regard, wall (or endothelial) shear stress (WSS) and plaque structural stress (PSS) may determine plaque development,

progression and rupture. WSS is the parallel frictional force exerted by blood flow on the endoluminal surface of the arterial wall. In principle, high (or physiological) WSS exerts atheroprotective property. However, supraphysiological (or very high) WSS, which occurs when blood flows through a stenotic atherosclerotic plaque, is associated with endothelial cell injury, inflammation and vascular remodeling. This further suggests that supraphysiological WSS is possibly involved with plaque rupture or thrombus generation, and can induce the erosion of the plaque surface. In addition, PSS is determined by plaque architecture and composition, as well as localisation, extent, and size of individual calcium deposits within the plaque. Increased PSS is associated with increased necrotic core thickness or size, as well as reduced fibrous cap thickness. High PSS levels that exceed the plaque's strength can damage fibrous caps and thus cause plaque rupture (see review ²⁵). Upon rupture, a pro-thrombotic necrotic core becomes exposed to circulating platelets, resulting in platelet aggregation (thrombus). Moreover, proinflammatory cytokines can also increase the expression of the potent procoagulant, tissue factor, which triggers thrombosis in the ruptured plaque ²⁶. Subsequently this leads to arterial blockage, which may result in MI or stroke ²¹. The loss of cardiac function due to ACS can eventually lead to HF.

HF, a complex clinical syndrome, is defined by a heart's inability to maintain workload, leading to oxygen and nutrient delivery that is insufficient to meet the requirements for tissue metabolism²⁷. HF is the final common stage of all cardiac diseases and considerably increases the risk for morbidity and mortality²⁸. HF can be categorized based on the left ventricular ejection fraction (LVEF), i.e. (I) HF with preserved EF (HFpEF, diastolic HF; normal LVEF; typically considered as $\geq 50\%$), (II) HF with mid-range (HFmrEF; LVEF 40-49%), and (III) HF with reduced EF (HFrEF, systolic HF; typically considered as $<40\%$) ²⁹. There are three causes of HF: dysfunctional myocardium (e.g. ischemic heart disease, drug-induced cardiomyopathy), prolonged abnormal loading conditions (e.g. hypertension, volume overload) and arrhythmias (e.g. tachyarrhythmia, bradyarrhythmias) ²⁹. MI secondary to ischemic heart disease is thought to be the most important risk factor for HF ³⁰. Although the risk of HF associated with hypertension is smaller than that associated with MI, hypertension contributes considerably to the population burden of HF, as it is much more frequent than MI ³¹. Left ventricular hypertrophy is both a major maladaptive response to chronic pressure overload and an important risk factor in patients with hypertension, which frequently progresses to HF ³². Importantly, cardiac endothelial cells (EC) directly modulate performance of the subjacent cardiomyocytes, resulting in control of the onset of ventricular relaxation, and rapid filling of the heart. Thus, damage and/or dysfunction of the cardiac endothelium could have a serious impact on the development of cardiac diseases ^{33, 34}.

Together, the major cause of morbidity and mortality worldwide is atherosclerotic vascular disease, leading to stroke, MI and HF. Importantly, cardiovascular function is mediated by vascular endothelium. Endothelial cell activation and dysfunction attribute to pathogenesis of cardiovascular diseases.

Vascular endothelium and cardiovascular diseases

EC form a continuous monolayer, lining the blood luminal surface of vessels. This was originally thought to be an inert cellophane-like membrane in the vascular tree, with no function other than the maintenance of vessel wall permeability^{35, 36}. However, EC are now recognized as a crucial player in maintaining a wide range of cardiovascular homeostatic functions. Indeed, EC regulate vascular tone and blood flow, through the release of vasodilators and vasoconstrictors; modulate hemostasis/thrombosis, through the secretion of pro- and anti-coagulant and fibrinolytic agents; mediate inflammatory responses, via release and reaction with various cytokines and adhesion molecules; generate angiogenic and angiostatic growth factors, and peptides, thus maintaining blood vessel homeostasis^{37, 38}.

In EC, endothelial nitric oxide synthase (eNOS) is the major source of NO, and is an important regulator of cardiovascular homeostasis. Normal function of eNOS requires dimerization of the enzyme and the presence of its cofactor tetrahydrobiopterin (BH4). A functional eNOS oxidizes its substrate L-arginine to L-citrulline and NO, the latter of which plays key role in cardiomyocyte contractility and vascular tone^{5, 39}. It is well documented that uncoupling eNOS is a prominent source of myocardial reactive oxygen species (ROS), contributing to pathogenesis of pressure overload-induced ventricular remodeling and cardiac dysfunction^{40, 41}. When eNOS uncouples and shifts from a dimer to a monomer state, it results in reduced NO production and increased ROS generation⁴². It has been suggested that BH4 deficiency is likely to represent the major cause for eNOS uncoupling⁴³. In addition, the stoichiometry of intracellular BH4/eNOS interactions as well as the ratio of BH4 and its oxidized form, dihydrobiopterin (BH2), are the key determinants of eNOS coupling⁴³.

Endothelial dysfunction is defined by EC losing their ability to maintain this delicate balance, shifting the endothelium from an anti-thrombotic, anti-inflammatory, and vasodilation-prone surface, to conditions inclined to coagulation, inflammation and vasoconstriction⁴⁴. The mechanisms underlying endothelial dysfunction involve decreased NO bioavailability, activation of the pathways of vascular smooth muscle contraction, vascular oxidative stress, and inflammation^{45, 46}. Additionally, decreased endothelium-dependent vasodilator capacity of coronary arteries in part contributes to reduced myocardial perfusion, and hence impaired ventricular function⁴⁷. Endothelial dysfunction is associated with the process of plaque formation and atherosclerosis

progression, resulting from increased vascular permeability, upregulation of adhesion molecules, cytokine secretion, leukocyte adherence, elevated oxidized-low density lipoprotein (oxLDL), platelet activation, and vascular smooth muscle cell (VSMC) proliferation and migration⁴⁵. EC have a crucial role in the recruitment and adhesion of leukocytes, whose infiltration into lesions is a prominent feature of atherosclerosis. In response to normal laminar flow condition, EC generate low amounts of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). However, production of adhesion molecules is sharply increased in non-laminar or turbulent flow⁴⁸. The flow disturbances that occur naturally, or are associated with vascular diseases and interventions, can lead to neointimal hyperplasia or thrombosis, causing pathological conditions, such as atherosclerosis, in-stent restenosis, and bypass graft occlusion³⁸.

The hallmark of endothelial dysfunction is impairment of NO mediated endothelium-dependent vasodilation⁴⁹. When endothelial dysfunction is present, the ability of the blood vessels to dilate and/or constrict can be assessed³⁵. Although, measuring a patient's endothelial function is not routinely done, assessments of endothelial function is a good predictor of future cardiac events in individuals at risk of CVD and those with established CVD^{50, 51}. Endothelial function can be assessed by measuring dilation in response to stimuli such as hyperemia or after pharmacological stimuli. Impaired vasodilatation would be indicative of poor endothelial function⁵¹. To distinguish between endothelial dysfunction and SMC dysfunction as a cause for the vasodilation impairment, endothelium-dependent and -independent vasodilatation (i.e. acetylcholine and sodium nitroprusside, respectively) are typically assessed⁵². Several approaches can be used to evaluate endothelial function, including (I) invasive macrovascular techniques (e.g. iontophoresis), (II) noninvasive macrovascular techniques (e.g. flow-mediated dilatation and glyceryl trinitrate), or (III) measurement of morphologic and mechanical characteristics of the vascular wall (e.g. carotid intima-media thickness)^{52, 53}.

General background of angiogenesis

As previously mentioned, EC function in maintaining blood vessel formation, is characterized by four parameters; (I) *de novo* primary vessel formation involving the migration, differentiation and incorporation of endothelial progenitors (vasculogenesis), (II) the sprouting of new blood vessels from the pre-existing ones (angiogenesis), in which endothelial cells are responsible for the capillary growth, migration and organization of the vessel lumen, (III) the increase in the size of the lumen of pre-existing arterioles by remodeling and growth (arteriogenesis), a process that is controlled by mural cells (i.e. pericytes and VSMC, and (IV) recruitment of bone marrow-derived cells and/or endothelial progenitor cells to the vascular wall for further

incorporation into the endothelial lining during vascular repair or expansion of pathological vessels in adult (postnatal vasculogenesis)⁵⁴.

When an existing vessel is stimulated with angiogenic signals, such as hypoxia, angiogenic factors (e.g. vascular endothelial growth factor (VEGF) -A, fibroblast growth factor (FGF)), or NO, it causes mural cells to detach from the vascular wall, and junctions between EC to loosen, resulting in increased vascular permeability. The extravasation of plasma protein allows the formation of a provisional matrix, onto which endothelial cells migrate. The microvascular sprout is guided by a specialized endothelial cell (tip cell), while the neighboring endothelial cell (stalk cell) proliferates to elongate the stalk. Ultimately, a vessel lumen is established, and mural cells are recruited to ensure neovessel stability⁵⁴. Once the new vessel sprout matures, EC maintain in a quiescent, non-proliferative and non-migratory, cobblestone-like phenotype, named phalanx cells⁵⁵. Subsequently, these EC become covered with pericytes, which suppress endothelial cell proliferation and release cell-survival signals such as VEGF-A and angiopoietin (Ang)-1. A basement membrane is then established⁵⁴. High vascular endothelial growth factor receptor (VEGFR)-1 levels and subsequent low VEGF-A responsiveness enable these cells to stay quiescent for years⁵⁵. EC display the remarkable capability to switch rapidly from a quiescent state to a highly migratory and proliferative state during vessel sprouting, in response to injury or in pathological conditions⁵⁵. This switch is mediated by the Ang-Tie system⁵⁴. Ang1 is predominantly expressed by mural cells, and in binding with its receptor Tie2, it has a role in maintaining vascular stability, maturity, and integrity as well as inhibiting VEGF-A induced permeability^{56,57}. In contrast, Ang2 is synthesized primarily by EC, and is traditionally thought to antagonize Ang1 signaling. Ang2 binds Tie2, allowing EC to respond to destabilizing and angiogenic signals. In addition, Ang2 generation is activated by hypoxia, VEGF-A, FGF, and various proinflammatory endothelial activators, and its expression is mainly restricted to sites of vascular remodeling⁵⁶⁻⁵⁸. Importantly, high levels of Ang2 have been observed in human atherosclerotic plaques, and are associated with increased plaque angiogenesis and potentially also with microvessel destabilization, suggesting increased leakage⁵⁹.

Angiogenesis is both an essential adaptive response to physiological stress and an endogenous repair mechanism after ischemic injury⁶⁰. Under physiological conditions, angiogenesis occurs during growth, physiological adaptation and tissue repair (e.g. gestation, exercise-induced skeleton and cardiac muscle hypertrophy, wound healing)⁶¹. Pathological angiogenesis on the other hand, contributes to chronic inflammation and cancer. Indeed, insufficient and excessive angiogenesis play determinant roles in the pathogenesis of MI and atherosclerosis, respectively.

The role of angiogenesis in myocardial infarction

In the healthy heart, a dense network of capillaries ensures sufficient oxygen supply to meet the high energy demand of properly contracting myocytes. During physiological (adaptive) cardiac growth, myocytes release angiogenic growth factors in response to hypertrophic stimuli, which in part regulate the balance between cardiac hypertrophy and angiogenesis. Disruption of coordinated myocyte growth and angiogenesis, leads to contractile dysfunction, and promotes the progression from adaptive cardiac hypertrophy to heart failure⁶². This suggests that heart growth and function are angiogenesis dependent. Interestingly, it has been reported that newly formed blood vessels may secrete factors that promote the growth of cardiomyocytes⁶³. Taken together, this suggests the crosstalk between the myocardium and the vasculature functions to promote the growth of each, in a paracrine fashion.

Myocardial ischemia is a primary cause for the loss of vital components such as cardiomyocytes in the heart, leading to MI and eventually cardiac dysfunction or HF⁶⁴. In addition, endothelial dysfunction and/or decreased angiogenesis cause inadequate oxygen supply. Subsequently, this can result in progressive myocyte loss, replacement fibrosis and infarct extension, as well as hypertension, due to increased systemic resistance^{65, 66}. Early after MI, cardiomyocytes, EC and inflammatory cells express hypoxia-inducible transcription factors, such as hypoxia-inducible factor 1 alpha (HIF-1 α), which can be expressed for up to 4 weeks after MI in rats⁶⁷. In addition, various proangiogenic factors are expressed in ischemic heart, of which the VEGF-A and FGF families are the most studied growth factors in post-MI angiogenesis. VEGF-A is rapidly induced in the ischemic heart, in both human and animal studies, suggesting that VEGF-A is a likely mediator of ischemia-induced myocardial neovascularization⁶⁸⁻⁷⁰. Immunohistochemical analysis of heart tissue from patients with early or evolving infarction, or cardiac ischemia revealed HIF-1 α expression in both myocardial and endothelial cells. In contrast, VEGF-A⁺ cells were found only in the endothelium that lined small to medium arterioles and capillaries in the myocardium, suggesting that the angiogenic effects of HIF-1 and VEGF-A are limited to regions of terminal small vessels in the myocardium⁶⁹.

In response to myocardial ischemia, HIF-1 α and HIF-2 α accumulate in cardiomyocytes and other cell types. HIF-1 transcriptional activation would upregulate angiogenic factor expression such as VEGF-A; however, the angiogenic capacity was seen to be suppressed rather than enhanced⁶⁴. A study of chronic pressure overload-induced cardiac hypertrophy in mice revealed that cardiac VEGF-A levels were increased in the early phase, but decreased in later phases of cardiac hypertrophy, and this downregulation was associated with suppressed myocardial angiogenesis⁷¹. Several studies have suggested that loss of copper, a HIF-1 cofactor, and

accumulation of p53, are partially responsible for inhibition of HIF-1 activity-mediated VEGF-A expression. This thereby impairs cardiac angiogenesis in chronic ischemic heart and sustained pressure overload-induced cardiac hypertrophy^{64, 71, 72}.

Therefore, maintenance of the microvasculature in a failing myocardium may preserve blood supply and preserve myocardial function. Angiogenesis has the potential to restore ischemic myocardium at early stages after MI, and is also essential to prevent the transition to HF through the control of cardiomyocyte hypertrophy and contractility^{70, 73}.

The role of angiogenesis in atherosclerosis

The VV are specialized microvasculature, and play a major role in normal vessel wall biology and pathology. Under physiological conditions, VV originate from the adventitia and provide oxygen and nutrient to arterial and venous walls^{74, 75}. In normal human coronary arteries, VV is present in the adventitia and outer media layer, but absent in the intima⁷⁶.

Plaque angiogenesis is a physiological response to cope with the increased oxygen demand in the plaque. It is proinflammatory and proatherogenic, and enhances plaque vulnerability⁷⁷. It has been suggested that neovascularization in early plaques may provide an additional route for leukocyte and lipid accumulation relative to the arterial lumen. Hypoxic areas are known to be present in human atherosclerotic lesions⁷⁸. During atherosclerosis, the intima increases in thickness, which results in regional limited oxygen exchange, and partly contributes to plaque hypoxia. Importantly, plaque macrophages, with their high metabolic demand, are a major contributor to hypoxia in human plaque. Hypoxic macrophages upregulate HIF-1 α which induces angiogenesis, leading to plaque progression⁷⁹. EC react to hypoxic conditions by becoming more angiogenic, apoptosis-prone, and proinflammatory. They also alter their metabolism - all processes that promote endothelial dysfunction and atherogenesis^{77, 80}. These effects may involve HIF-1 α , a master regulator of hypoxic responses^{81, 82}. In early human plaque, the density of adventitial VV is much higher than that of intraplaque VV⁷. Most of the intraplaque VV are endothelialized, and only a few have mural pericytes and vascular smooth muscle cells. Lack of mural cells, poorly formed endothelial cell junctions, and the close proximity of inflammation, probably induce leakiness of the intraplaque VV^{7, 76}. In addition, VV density is correlated with atherosclerosis progression and morphology of atherosclerotic lesions⁷, as well as with intraplaque hemorrhage and plaque rupture⁷⁶. Therefore, it is fair to assume that functional aberrations of plaque angiogenesis causes an exacerbation of the atherosclerotic process.

General background of lymphangiogenesis

Under physiological conditions, excess interstitial fluid, stemming from leaky endothelium is drained via lymphatic vessels. Lymphatic vessels were first officially described in the 1600s, and named, after their milky fluid content: *venae albae aut lacteae* (lacteal vessels)^{83, 84}. In the late 1990s, the identification of molecular markers of lymphatics^{84, 85}, enabled researchers to investigate the (patho) physiological role of lymphatics and the molecular mechanisms of their formation in closer detail. Next to fluid homeostasis, lymphatic vessels have important functions in lipid absorption, and immune surveillance^{86, 87}, whilst recent studies have suggested additional functions of the lymphatic vasculature in fat metabolism, obesity, inflammation, and the regulation of salt storage in hypertension^{87, 88}.

The lymphatic vascular network consists of thin-walled, blind-ended capillary-sized vessels, called “initial lymphatics”, which serves for interstitial fluid absorption and its transport to “pre-collectors”, with the final drainage via “collecting lymphatics”^{89, 90}. Initial lymphatics are hallmarked by LYmphatic VEssel hyaluronan receptor 1 (LYVE-1)-expressing lymphatic endothelial cells (LEC), the absence of SMC and the lack of pericyte coverage. Pre-collectors contain valves and have sparse smooth muscle cell coverage. Collecting lymphatics are characterized by podoplanin (PDPN) expression, with absent, or only very weak LYVE-1 expression, and are covered with continuous basement membrane and SMC. Collecting lymphatics are segmented by unidirectional valves into units called lymphangions^{91, 92}. This contractile unit propels lymph into the next lymphangion through the interposed valves, in a unidirectional manner⁹³. These collecting lymphatics transport the protein-rich lymph to the proximal site, and return the lymph to the cardiovascular system via the thoracic duct.

Lymphangiogenesis is the formation of new lymphatic vessels from pre-existing lymphatics⁹⁰, essential in embryonic development. In adults, lymphangiogenesis is actively involved with pathological processes, including inflammatory diseases and tumor dissemination. In addition, insufficient lymphangiogenesis occurs in lymphedema, which is characterized by chronic tissue edema and impaired immunity^{86, 90}. The process of lymphangiogenesis is thought to be similar to angiogenesis, during which quiescent LEC become activated to degrade their surrounding extracellular matrix, directionally migrate towards the lymphangiogenic stimuli, proliferate and organize into new capillary networks^{85, 94, 95}. It has been suggested that at cellular level, lymphangiogenesis also has similarities to angiogenesis, as it is characterized by tip and stalk cell differentiation, and involves similar regulators such as VEGF-C, neuropilin-2 (NRP2), Notch homolog 1, translocation associated (Drosophila); also known as Notch1, and Delta-like 4 (DLL4)^{85, 96}.

During embryogenesis, the formation of lymphatics is initiated by the transcription factors; sex determining region Y box 18 (SOX18) / chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), inducing prospero homeobox 1 (PROX1) expression in a subset of venous EC (VEC) of the cardinal vein. Venous PROX1-expressing EC (referred to LEC progenitors) migrate away from the cardinal vein. This process requires the graded expression of VEGF-C in the surrounding mesenchyme. The budding LEC maintain the expression of VEGFR-3 and begin expressing PDPN, indicating that lymphatic differentiation is progressing. Subsequently, LEC assemble together and establish lymph sacs (primordial thoracic duct). Following LEC proliferation and sprouting, the majority of the lymphatic network arises from these sacs⁹⁷. Lymphatic vessels undergo further remodeling and maturation, including valve formation; processes mediated by Ang2, PDPN, ephrin-B2 and Foxc2 (forkhead box C2), subsequently establishing a functional lymphatic vessel network⁹⁸.

During adulthood, the majority of lymphatic vessels are quiescent, with the exception of reproductive organs during the ovarian cycle and gestation; however, lymphangiogenesis is often activated in several pathological conditions^{99, 100}. Adult lymphangiogenesis occurs primarily by sprouting from pre-existing vessels, although bone marrow-derived cells, such as macrophages, may transdifferentiate into lymphatic endothelium in some pathological conditions^{88,90}. Importantly, physiological and pathological lymphangiogenesis likely share certain biological mechanisms, i.e. activation of the VEGF-C/VEGFR-3 signaling pathway^{100,101}. Additionally, pathological lymphangiogenesis is also mediated by proinflammatory cytokines and/or growth factors such as VEGF-A. Moreover, activated macrophages are a major source of VEGF-C and/ or VEGF-D, and induce lymphangiogenesis during inflammation and tumor development^{88, 100}.

In summary, the mechanisms regulating lymphangiogenesis are poorly understood, when compared to angiogenesis. Physiological and pathological lymphangiogenesis is mediated by, in part, a similar molecular signaling pathway to that governing angiogenesis. Recent research has implicated the lymphatic system in the pathogenesis of cardiovascular diseases, including obesity and metabolic disease, dyslipidemia, inflammation, atherosclerosis, hypertension, and MI⁹³.

Role of lymphangiogenesis in myocardial infarction

A pioneering investigation of the cardiac lymphatic system was performed in the 17th century by Rudbeck, who described lymphatics in the dog heart^{102, 103} however, cardiac lymphatics have been relatively neglected in cardiovascular research for decades. Nevertheless, cardiac lymphatics have gained interest recently, as increasing evidence has revealed the important role of cardiac lymphatic

vasculature in maintaining normal heart function^{104, 105}. Cardiac lymphatics function in draining myocardial interstitial fluid and thus play a role in controlling tissue pressure, and help to prevent myocardial edema¹⁰⁶. In the adult heart, cardiac lymphatics are located most abundantly in the ventricles¹⁰⁷. Although there are some species-specific anatomical variations, cardiac lymphatics of most species consist of (1) terminal capillaries, (2) a capillary plexus penetrating the subendocardium, myocardium, and the subepicardium, and (3) collecting vessels that lead the lymph out of the heart^{108, 109}. In animal studies, acute cardiac lymphatic obstruction caused epicardial lymphedema and lymphangiectasis. Also, chronic obstruction led to myocardial edema, subendocardial hemorrhages, endocardial fibrosis and myocardial fibrosis primarily involving the left ventricle¹⁰⁶. In humans, impairment of lymph flow is associated with coronary artery injury¹⁰⁵. Obstruction of cardiac lymphatics takes place during open-heart surgery and can cause atrial fibrillation, prolonged pericardial or pleural effusions, also known as “swollen heart”¹¹⁰. Only a few studies have demonstrated the role of lymphatic vessels in MI. A recent study has demonstrated that adverse remodeling of epicardial collector lymphatics in the infarcted area causes reduced cardiac lymphatic flow and persistent myocardial edema in rats¹¹¹. In response to MI, cardiac lymphatics substantially expanded in rodents^{111, 112} as well as in postmortem human MI samples¹¹³. Intraperitoneal injection of VEGF-C 156S¹¹² or treatment with microparticle-based intramyocardial delivery VEGF-C 156S protein¹¹¹ promoted post-MI lymphangiogenesis, resulting in improved cardiac function and attenuated cardiac inflammation in rodent MI models. This suggests the beneficial effect of pro-lymphangiogenic therapy in MI.

Role of lymphangiogenesis in atherosclerosis

Lymphatic functions are related to a causal role in atherogenesis, including immune response and cholesterol metabolism⁴. Lymphatic vessels were first described in human aortic walls by Johnson in the late 1960s¹¹⁴. Lymphatics can be observed in the arterial wall of a variety of animals (e.g. rabbits, dogs, rats and mice) and seem to be mostly present in the adventitial layer⁴. In human atherosclerotic arteries, lymphatic vessels were reported previously to exist predominantly in the adventitia^{115, 116} while they are rarely present in the intima¹¹⁷. However, Kholova et al.¹¹⁶ have reported that lymphatics also are detectable in media and intima of progressive human coronary atherosclerotic lesions. This concurs with recent findings showing abundant lymphatics within the atherosclerotic lesion of human carotids⁴. In human atherosclerotic lesions, the number of adventitial lymphatics is correlated with disease progression¹¹⁵ and lymphatic dysfunction is correlated with atherogenesis¹¹⁸. Plaque lymphangiogenic vessels were often collapsed and seemed mostly associated with areas rich in extracellular matrix, whereas more inflamed areas displayed mostly angiogenesis¹¹⁶. Dilatation of adventitial lymphatics has been observed in animal¹¹⁹

and human¹¹⁶ atherosclerotic lesions, possibly as a result of an increased arterial wall permeability. Moreover, adventitial VV that expands in plaque lesion are often dysfunctional and leaky, potentially causing interstitial fluid buildup and local edema^{120, 121}. This may be similar to the lymphatic remodeling that has been observed in cancer, where lymphatics are often collapsed and enlarged due to the excessive interstitial pressure and edema, implying lymphatic dysfunction^{85, 122}. Interestingly, upon growth of the plaque and increasing plaque interstitial pressure, the LEC elongate, and this stretching activates $\beta 1$ integrin signaling mediated VEGFR-3 phosphorylation and subsequent induction of lymphangiogenesis¹²³.

In addition, the functional consequences of adventitial lymphangiogenesis for atherosclerosis are largely unknown. In 1970, Jellinek et al.¹¹⁹ proposed that sufficient lymphatic drainage was required for transporting excess plasma accumulation in aortic atherosclerotic lesions, and that disturbance of lymph drainage contributed to lipid accumulation, resulting in the aggravation of arteriosclerotic lesions. During 1970s to 1980s, it was further suggested that the integrity of the lymphatic system draining the coronary arterial wall, might have an important role in the lipid efflux mechanism and the occurrence of atherosclerosis in humans¹¹⁸. More than 40 years ago it was postulated that the ingrowth of VV occurred in response of metabolic demand within arterial walls, and thus increased passage of materials to arterial walls. However, ingrowth of lymphatics did not occur in the media or intima, and subsequently led to lipid accumulation within intimal plaque due to the lack of escape route¹²⁴. Recent studies have demonstrated that plaque lymphangiogenesis functions in lipoprotein metabolism¹²⁵ and cholesterol clearance¹²⁶. Moreover, lymphatic dysfunction precedes the onset of atherosclerosis^{120, 127}. Disruption of lymphatic drainage accelerates plaque progression due to cholesterol retention¹²⁸ and T-cell accumulation¹²⁰ in plaque lesions. Moreover, plaque lymphangiogenesis could exert atheroprotective functions by draining plaque-contained lipids during regression¹²⁸. Systemic treatment with VEGF-C 152s, a selective VEGFR-3 agonist, improved lymphatic cellular transport in pre-atherosclerotic mice¹²⁷.

Together, it is now becoming evident that the lymphatic system plays an active role in cardiovascular disease, and lymphatic dysfunction accelerates disease progression. Sufficient lymphatic drainage seems to protect against MI and atherosclerosis. However, many aspects of the lymphatic system remain unclear, and future studies are needed to fully understand the mechanism of lymphangiogenesis in cardiovascular disease. Recent advances in lymphatic research will potentially provide novel insights into the treatment of cardiovascular diseases.

Overall aim and hypothesis

The general aim of this thesis is to investigate novel endothelium-targeted therapeutic approaches for pressure overload-induced heart failure, and for controlling lymphangiogenic functions in atherosclerosis.

We hypothesize that:

1. AVE3085, an eNOS transcription enhancer, can protect against pressure overload-induced left ventricular hypertrophy.
2. Gene network analysis will yield co-expressed genes correlating with human plaque lymphatic vessel density, which are be critical in the regulation of lymphangiogenesis-associated atherosclerosis.

Outline of the thesis

The pathogenesis of myocardial and endothelial dysfunction shows an important role for eNOS uncoupling. BH4 is an essential cofactor for eNOS, and decreased BH4-bioavailability, resulting in subsequent uncoupling of eNOS. In **chapter 2** we discussed the pathophysiologic role of decreased BH4 bioavailability, molecular mechanisms regulating its metabolism, and its potential therapeutic use, as well as its pitfalls as an NOS-modulating drug. **Chapter 3** reviewed the important role of eNOS uncoupling in the pathogenesis of endothelial dysfunction, and the potential therapeutic interventions to modulate eNOS for tackling endothelial dysfunction. Pharmacological approaches that prevent eNOS uncoupling are of therapeutic interest. Hence, in **chapter 4** we discussed modulation of eNOS activity by novel small molecules AVE9488 and AVE3085, which concomitantly enhanced eNOS transcription and reversed the eNOS uncoupling. To investigate the potential protective effect of elevated eNOS expression in ventricular hypertrophy, in **chapter 5** we investigated the protective effects of AVE3085. We determined whether eNOS modulation could reverse cardiac hypertrophy and dysfunction in pressure overload-induced heart failure model. We subjected mice to severe transverse aortic constriction (TAC) for 1 week, as a model of acute onset of HF. It is well established that eNOS uncoupling plays a major role in TAC pathobiology. Moreover, mice that underwent severe, but not mild, TAC resulted in enhanced eNOS uncoupling, associated with increased eNOS-dependent oxidative stress, and consequent cardiac dysfunction and remodeling^{129, 130}. We used C57BL/6 mice because they develop rapid cardiac hypertrophy, myocyte hypertrophy and fibrosis within 7 days after TAC^{131, 132}, allowing us to examine the utility of pharmacological or molecular interventions that may limit hypertrophy.

As discussed, another effective method to prevent the deleterious effects of vascular leakage is to induce the drainage of leaked fluids via lymphatics; as lymphatic vessels are pivotal for drainage of immune cells and lipids. As inflammation and lipid metabolism are causally related to atherogenesis, in **chapter 6**, we investigated the process of lymphangiogenesis and its main drivers in human atherosclerosis. This was done using a new systems medicine approach, where we correlated microarray data from stable and ruptured atherosclerotic lesions to plaque lymphangiogenesis to identify candidate genes. Subsequently, we performed deep bioinformatics interrogation of expression array data and *in vitro* loss-of-function screening to identify novel genetic regulators of lymphangiogenesis-associated atherosclerosis. Finally, **chapter 7** summarized and discussed the major findings of this thesis and future perspectives.

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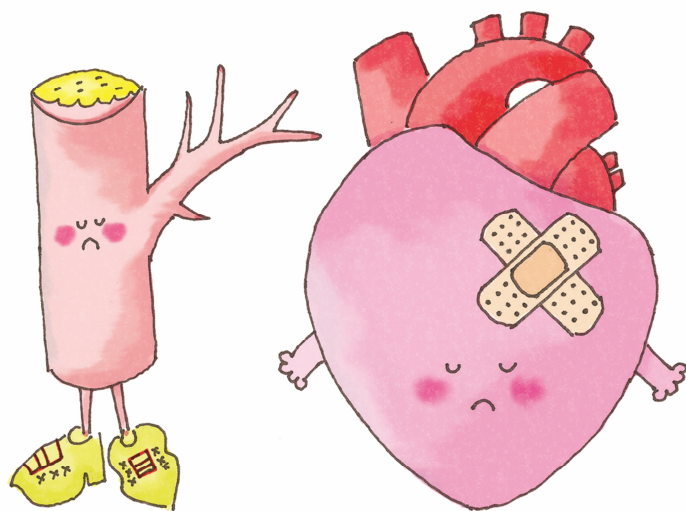
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Chapter 2

Targeting endothelial and myocardial dysfunction with tetrahydrobiopterin

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Abstract

Tetrahydrobiopterin (BH4) is an essential cofactor for aromatic amino acid hydroxylases and for all three nitric oxide synthase (NOS) isoforms. It also has a protective role in the cell as an antioxidant and scavenger of reactive nitrogen and oxygen species. Experimental studies in humans and animals demonstrate that decreased BH4-bioavailability, with subsequent uncoupling of endothelial NOS (eNOS) plays an important role in the pathogenesis of endothelial dysfunction, hypertension, ischemia/reperfusion injury, and pathologic cardiac remodeling. Synthetic BH4 is clinically approved for the treatment of phenylketonuria, and experimental studies support its capacity for ameliorating cardiovascular pathophysiologies. To date, however, the translation of these studies to human patients remains limited, and early results have been mixed. In this review, we discuss the pathophysiologic role of decreased BH4 bioavailability, molecular mechanisms regulating its metabolism, and its potential therapeutic use as well as pitfalls as an NOS-modulating drug. This article is part of a special issue entitled “Key Signaling Molecules in Hypertrophy and Heart Failure.”

1. Introduction

Tetrahydrobiopterin (BH4) is an essential cofactor for the three aromatic amino acid hydroxylase enzymes involved in the synthesis of neurotransmitters, as well as the nitric oxide synthase (NOS) isoforms. In the cardiovascular system, the NOS isozymes have a central role in mediating vascular tone, endothelial function, platelet aggregation, cardiac myocyte contraction and excitation–contraction coupling, and many other properties. NOS activity depends on several cofactors, including flavin adenine dinucleotide, flavin mononucleotide, a heme group, and BH4, as well as structural features such as a zinc–thiolate cluster. BH4 facilitates electron transfer from reductase to oxidase NOS domains, coupling this to the conversion of arginine to citrulline and NO generation. It also promotes and stabilizes NOS in its active homodimeric form¹. Decreased BH4 bioavailability results in unstable NOS that becomes physically less compact and functionally uncoupled, reducing NO-production and enhancing the generation of superoxide. Though the ratio between “coupled” and “uncoupled” NOS in normal or pathological disease conditions remains unclear, uncoupling can be reversed by exogenous BH4 administration, igniting interest in BH4 as a potential cardiovascular therapeutic. Both a reduced bioavailability of the substrate L-arginine and the cofactor BH4 can lead to uncoupling of eNOS. However, in *in vivo* settings the latter is the most prominent factor. Recoupling of eNOS accounts for the majority of the benefits that result from BH4 administration in a model of pressure overload. Moens et al.² have demonstrated that administration of the potent antioxidant Tempol (which did not recouple the uncoupled eNOS), did not have any significant effect on remodeling, except on myocyte dimensions (but still less than the effect of BH4 on myocyte dimensions).

However, despite encouraging experimental data, clinical translation studies remain limited, and those reported (largely preliminary data) have been less than encouraging. The basic science continues to drive efforts to understand the biochemistry better, and ultimately identify a pathway to successfully adjust decreased BH4-levels. Here, we review key background regarding BH4 chemistry and physiology, and highlight new insights and controversies in this area of research.

1.1. Regulation of BH4 synthesis

BH4 is synthesized by one of the two pathways, *de novo*, or salvaged from its oxidized forms. For *de novo* synthesis, the enzymes GTP cyclohydrolase (GTPCH), 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase convert GTP into BH4³.⁴ GTPCH is the rate limiting enzymatic step and primary regulator for new synthesis (overview see Figure 1). GTPCH colocalizes with eNOS in caveolae, and upregulation of one enzyme requires matching upregulation of the other in order to maintain normal

NOS-NO synthesis⁵. For example, eNOS overexpression in the absence of compensating GTPCH levels leads to excessive reactive oxygen species (ROS) generation by NOS rather than enhanced NO production⁵. Salvage of BH4 from BH2 is achieved by dihydrofolate reductase (DHFR), or by quinonoid dihydrobiopterin through dihydropteridine reductase (DHPR). DHFR is mainly involved in folate metabolism but also converts inactive BH2 back to BH4 and plays an important role in the metabolism of exogenously administered BH4. A study in bovine aortic endothelial cell (BAEC) culture showed that angiotensin II (AT2) downregulates DHFR expression via endothelial NADPH-oxidase-derived H₂O₂, resulting in reduced NO production due to BH4 deficiency and the uncoupling of eNOS⁶. Overexpression of DHFR restores all of these abnormalities⁶. Seujsange et al. demonstrated that renal ischemia/reperfusion injury significantly reduced renal DHFR mRNA and protein levels which were restored by administration of ACE-inhibitor or AT2 receptor type 1 blocker (ARB)⁷.

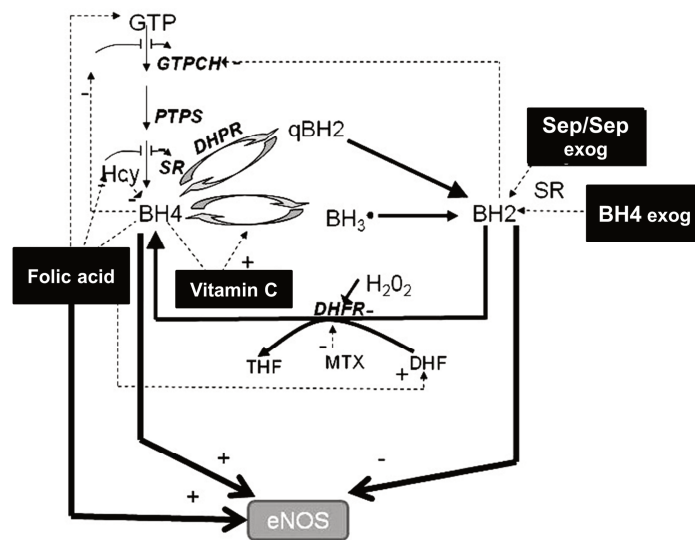


Figure 1. Biosynthesis of BH4

1.2. NOS uncoupling

The stoichiometry of intracellular BH4/eNOS interactions (or for that matter BH4 with other NOS isoforms) remains poorly defined, making it unclear whether intracellular BH4 deficiency alone is sufficient to induce eNOS uncoupling. Crabtree et al.⁸ found that the ratio of BH4:BH2 and absolute molar concentration of BH4 are the key determinants of eNOS coupling *in vivo*. Furthermore, they showed that eNOS:BH4 ratio and biopterin redox status are responsible for determining the degree of eNOS coupling even in the absence of vascular disease or oxidative stress. The degree of BH4 oxidation, BH2 accumulation, and superoxide production directly correlated with intracellular eNOS:BH4 ratio. BH4 can have substantial effects on the levels of cellular reactive oxygen species production through mechanisms independent of eNOS, such as direct reactive oxygen species scavenging. This suggests that general antioxidant properties of BH4 act in direct scavenging of superoxide and maintenance of intracellular redox balance. Moreover, they demonstrate that eNOS-dependent superoxide production occurs in addition to basal superoxide formation overwhelming antioxidant defenses. This is surprising as superoxide reacts with BH4 *in vitro* with a rate constant $>10,000$ -fold slower ($3.9 \times 10^5 \text{ mol l}^{-1} \text{ s}^{-1}$) than its near diffusion limited reaction with NO ($6.7 \times 10^9 \text{ mol l}^{-1} \text{ s}^{-1}$)^{9, 10}.

The role of BH2 in the pathogenesis of eNOS uncoupling has recently been elucidated. Before, BH2 was described as an inactive and oxidized form of BH4. Because the K_m of BH4 and BH2 for eNOS are similar ($\sim 80 \text{ nm}$), earlier studies speculated that BH2 competed with BH4 for eNOS binding, thus promoting NOS uncoupling and O_2^- production¹¹. This was recently shown, and the fall in BH4:BH2 ratio, rather than decline in absolute BH4, determined NO and O_2^- production by eNOS¹². Thus, strategies to augment BH4 while suppressing BH2 may be required to optimally achieve therapeutic benefit.

Although GTPCH is the key regulator of the total amount of intracellular biopterins, DHFR is critical to eNOS function as it determines BH4:BH2 ratio and thus eNOS coupling. In particular, DHFR is important in preventing “self-propagated” eNOS uncoupling where there are low total biopterin levels, and eNOS-dependent oxidation of BH4 that would further exacerbate this state can be rescued by DHFR¹³. Inhibition of DHFR activity or reduction of DHFR protein (by methotrexate or DHFR-specific siRNA, respectively) resulted in BH4 oxidation to BH2, reducing NO generation and increasing eNOS-derived O_2^- . This was particularly effective if BH4 was reduced. Sugiyama et al.¹⁴ showed in BAEC that while GTPCH knockdown reduced overall biopterin levels, lowering eNOS-NO synthesis, it did not enhance superoxide production. In contrast, DHFR knockdown yielded a marked increase in BH2, though no substantial effect on total biopterin, reducing NO generation while greatly

enhancing ROS production. These data suggest that decreased NO production and increased ROS production are not intrinsically linked by BH4 depletion. Even if BH4 levels are lowered to the point of uncoupling NO synthesis, BH2 appears necessary to observe eNOS-dependent H₂O₂ synthesis. Taken together, these data imply that BH2 plays a key role in generating ROS from eNOS in cultured endothelial cells, and the ratio of BH4:BH2, rather than the absolute concentrations is the critical determinant.

Recently, it was suggested that BH4 also has a role in mediating cardiac mitochondrial NOS (mtNOS), though the exact identification of a mtNOS species remains controversial. Using permeabilized cat ventricular myocytes, Dedkova et al. showed that BH4 reduced mitochondrial ROS generation and mitochondrial permeability transition pore opening while increasing mitochondrial NO generation ¹⁵.

1.3. BH4 and vascular pathophysiology

Endothelial dysfunction is defined by inability of endothelium to maintain vascular tone, increased platelet activation, leukocyte adhesion, and smooth muscle proliferation; and all can be linked to inadequate NO generation ⁵. Patients with diabetes, heart failure, and hypertension, each conditions with excessive endothelial ROS, exhibit improved endothelium-dependent vasodilation after BH4 supplementation ¹⁶ compared with general antioxidants. In smokers, endothelial dysfunction is partially due to increased lipid peroxidation and the increased formation of oxidized low-density lipoprotein (oxLDL). OxLDL inhibits the Akt survival pathways attenuating eNOS activity ¹⁷⁻¹⁹. One study showed that cigarette smoke extract reduced eNOS activity in a concentration-dependent fashion ²⁰ due to BH4 depletion, possibly from BH4 oxidation ²¹. Human studies have shown that BH4 administration via intra-arterial infusion at a dose of 500 µg/min for 30 min ²⁰, or oral administration of sapropterin hydrochloride at a dose of 2 mg/kg ²², improves endothelial dependent relaxation in smokers. Administration of BH4 has also been described to eliminate accelerated atherosclerosis by recoupling eNOS in atherogenic apoE-deficient mice ²³.

Hyper-homocysteinemia has been emerged as an independent risk factor for atherosclerosis and vascular disease. Homocysteine diminishes the bioavailability of BH4 *in vitro*, and incubation of human umbilical vein endothelial cells with BH4 attenuates homocysteine-induced altered NOS activity and increased superoxide production ²⁴.

Intra-arterial BH4 improves endothelial dysfunction in patients with hypercholesterolemia²⁵. Cosentino et al. tested chronic oral BH4 (400 mg bid x4 weeks) showing its reversed endothelial dysfunction and oxidative stress in hypercholesterolaemia. Hph-1 mice that have 90% deficiency in GTPCH develop hypertension^{26, 27} and accelerated atherosclerosis²⁸, and this can be ameliorated by exogenous BH4 administration²⁹.

NOS is activated by shear stress, and recent studies have shown that BH4 biosynthesis is commensurately upregulated by this mechanical stimulation^{30, 31}. Li et al. revealed a mechanism for this response, showing that laminar shear caused dissociation of GTPCH from its inhibitory co-protein GFRP, and promoted GTPCH activation by phosphorylation via casein kinase2 (CK2)^{31, 32}, resulting in enhanced NO generation³². Oscillatory shear stress that generated no net shear had no impact on GTPCH-GFRP association, preventing GTPCH phosphorylation and leading to relatively reduced BH4 levels^{31, 32}.

Angiotensin (AT) type 1 receptor blockade improves eNOS coupling and reduces ROS production³³, while AT2 itself stimulates hypertension in part by ROS generation via eNOS derived O_2^- production⁶, an effect suppressed by administration of BH4. Endothelial NADPH oxidase-derived H_2O_2 downregulates DHFR expression that can impact BH2 levels, worsening eNOS uncoupling⁶. Recently, Xu et al.³⁴ found BH4 levels declined in endothelial cells after exposure to low concentrations of AT2, associated with tyrosine nitration of PA700 and accelerated GTPCH degradation.

Compared to the normotensive controls, spontaneous hypertensive rats have reduced plasma BH4 levels^{35, 36}, and BH4 administration blunts the development of hypertension, improving endothelial function by increasing eNOS NO versus O_2^- formation. In a low renin hypertension model, the DOCA-salt mouse, increased NADPH oxidase-derived superoxide was linked to increased BH4 oxidation, and oral BH4 supplementation reversed eNOS uncoupling and blunted hypertension. This did not occur in controls, showing how BH4 might have vasodilating efficacy but only if eNOS uncoupling was present³⁷. This could impact translation to patients, since the diagnosis of eNOS uncoupling is difficult, so identifying responsive patients may be tricky. An overview of all relevant preclinical studies (vascular and myocardial dysfunction) and clinical trial is given in Table 1 and 2.

2. BH4 and cardiac pathophysiology

While the role of BH4 and NOS uncoupling in the vasculature was well established, as recently as 2005, little was known as to its relevance for myocardial remodeling and dysfunction in heart disease. Takimoto et al.³⁸ first revealed that

eNOS was indeed an important source of myocardial ROS in a mouse model of pressure overload-induced by transverse aorta constriction. Mice developed myocardial and myocyte hypertrophy, interstitial fibrosis, and eventually cardiac dilatation and dysfunction. These changes occurred with concomitant oxidative stress generated largely and perhaps surprisingly from eNOS uncoupling. BH4 levels declined along with NOS-derived NO in hypertrophied myocardium. Mice lacking eNOS actually did better, with little oxidative stress and associated chamber dilation. Administration of BH4 to control mice improved the outcome, whereas this did not occur by administering the equally anti-oxidative yet non-NOS interactive tetrahydroneopterin. Moens et al. subsequently performed a more clinical translational study, showing that BH4 reversed hypertrophy, fibrosis, and chamber dysfunction, and suppressed myocardial ROS in mice in which pressure overload remodeling was already well established ². More recently, Silberman et al. ³⁹ reported that eNOS uncoupling may have a pathogenetic role in the diastolic dysfunction in mice with mild hypertension. This was ameliorated by BH4. Many heart failure patients have hypertrophy and diastolic dysfunction despite an apparently normal EF, and this study proposed BH4 as a potential treatment.

NOS uncoupling and altered BH4 biology have been identified as contributors to cardiac ischemia/reperfusion injury, though the focus has primarily been on vascular more than myocardial effects. Chronic BH4 improved functional recovery after global ischemia/reperfusion by preventing coronary endothelial dysfunction, decreasing malondialdehyde (MDA), a product of lipid peroxidation and index of tissue injury ⁴⁰, restoring NOS-NO production and improving postischemic recovery of NOS-dependent coronary flow ⁴¹. Other studies have shown BH4 improved contractile and metabolic abnormalities ^{42, 43} post reperfusion, and reduced polymorphonuclear cell adhesion and tissue infiltration possibly by inhibiting inter-cellular adhesion molecule-1 expression ^{44, 45}. The role of NOS uncoupling in ischemic myocardium itself has not yet been clarified to date.

2.1. Is there translational potential for BH4?

As already noted, many small clinical trials have appeared supporting benefits of BH4 treatment in disorders involving vascular and in particular endothelial disease ⁴⁶. With the development of synthetic sapropterin hydrochloride (6R-BH4, Kuvan®), larger trials were feasible with an FDA approved formulation, and hope was that similar efficacy would be observed. 6R-BH4 was studied in a number of phase I–II clinical trials for diseases including arterial (NCT00325962, concluded 2008) and pulmonary hypertension (NCT00435331, recruitment stopped in 4/09, no data reported), endothelial dysfunction (NCT00532844, study completed, data unpublished), peripheral vascular disease (NCT00403494, completed 1/09) and sickle

cell disease (NCT00445978, completed 6/09). The results were disappointing, not because they revealed adverse effects, but for their lack of efficacy. There has been some evidence of improved endothelial function in sickle cell patients⁴⁷, though the primary goal of reducing episodes of sickle cell crisis was unaltered. Given this history, clinical assessment of this particular compound for cardiovascular indications appears to have come to a halt.

What happened? There are many reasons by which one could hypothesize that administering excess oral BH4 might not be effective. If the stoichiometry with NOS isoforms has to be just right, too much might activate constitutive NOS (iNOS) in settings of inflammation, exacerbating pathophysiology. This has been observed in ischemic brain injury, where BH4 levels, GTPCH activity, and iNOS activity rise within 24 h due to action of proinflammatory cytokines, resulting in excessive NO production, increased peroxynitrite, and neuronal damage⁴⁸. The human pathology is far less clean and uniform than that generated in animal models. Secondly, the relationship between plasma and target tissue BH4 levels remains unclear. BH2 can be more efficiently transported into cells over BH4, but must then be recycled back to BH4⁴⁹. Oral BH4 is likely oxidized and must be re-reduced, so this biochemistry becomes very important for the net result. Some efforts have been made to enhance this re-reduction process by combining BH4 with anti-oxidants such as vitamin C, though evidence for clinical efficacy of this combination remains lacking. Recently, Suckling et al.⁵⁰ revealed a novel BH4 analog 6-acetyl-7,7-dimethyl-5,6,7,8-tetrahydropterin (ADDP), a stable compound soluble in both polar and organic solvents. ADDP can diffuse from the plasma, across cell membranes, and cause vasodilatation by stimulating eNOS activity. This may provide another avenue for clinical translation.

In addition, the bioavailability of BH4 can be increased on several ways. Statins have been described to increase BH4 levels in vascular endothelial cells by potentiating GTPCH gene expression and BH4 synthesis, thereby increasing NO production and preventing relative shortages of BH4⁵¹. Another way to increase the bioavailability of BH4 in the vasculature has been the administration of folic acid. Folic acid increases the bioavailability of BH4 by (i) ameliorating the binding affinity of BH4 to eNOS, (ii) enhancing the regeneration of BH4 from the inactive form BH2 and (iii) chemically stabilizing BH4⁵². Furthermore Hyndman et al.⁵³ demonstrated that 5-MTHF is capable of binding the active site of nitric oxide synthase and mimicking the orientation of tetrahydrobiopterin.

While the actual preclinical studies regarding the effect of BH4 on myocardial and endothelial dysfunction are promising, some extra topics need to be investigated before extrapolation to the clinic takes place. There is a need to determine the dose–response relation between BH4 and its effect on superoxide generation in a model of

heart failure because of the tight stoichiometric relation between eNOS and BH4, with possible subsequent uncoupling. In addition, the uptake of BH4 and other eNOS modulators by myocytes of the failing or ischemic heart needs to be explored.

3. Summary

The biochemistry, molecular biology, and experimental translational evidence supporting an important role of BH4 to heart and vascular health by means of stabilizing NOS function and suppressing NOS-derived ROS seem compelling. Yet, the data also point to potential pitfalls that could easily limit the efficacy of this approach in clinical diseases. Most importantly, the instability of BH4 and thus likely requirement for robust intracellular re-reduction and the likely loss of benefit if this is not done just right, may have stymied recent efforts to test the translatability of BH4 supplementation for clinical disease. Whether alternative forms that cannot be oxidized, and/or are not dependent upon other enzyme systems to maintain stability can be generated and would work better is a question that remains open.

A major advantage is the fact that BH4 is already FDA approved as a therapeutic for the metabolic disorder phenylketonuria (PKU), a deficiency of phenylalanine hydroxylase that can be partially offset to by providing excessive BH4. Sapropterin dihydrochloride (Kuvan®, BioMarin, Tiburon, CA) is the synthetic version of BH4 that is currently used to treat PKU patients, and in many, the therapy lowers blood phenylalanine levels independent of dietary intake.

Given the many impressive results of BH4 as a new therapeutic strategy to tackle myocardial and endothelial dysfunction, one would hope that such efforts will continue, and some ultimate translational potential will be realized.

Table 1. Overview of major vascular and myocardial preclinical *in vivo* studies with BH4.

	Model	Major finding	Reference
Vascular dysfunction	apoE-KO/eNOS-Tg mice	BH4 reduced atherosclerotic lesion size by improvement of eNOS dysfunction apoE-KO/eNOS-Tg mice.	23
	Hph-1 mice	<i>In vivo</i> BH4 deficiency facilitates neointimal formation after wire-induced femoral artery injury. BH4 prevented vascular injury-induced increase in neointimal formation	54
	Spontaneously hypertensive rats	BH4 blunts oxidative stress	5
	Deoxycorticosterone acetate-salt (DOCA-salt) hypertension-mice	Increased NADPH oxidase-derived superoxide was linked to increased BH4 oxidation and BH4 supplementation reversed eNOS uncoupling and blunted hypertension	37
Myocardial dysfunction	Mice with <i>de novo</i> pressure overload	Uncoupling of eNOS due to decreased BH4 bioavailability is a major cause of ROS-generation in pressure overload- induced heart failure. Administration of BH4 from the start of pressure overload blocks the onset of ventricular remodeling	38
	Mice with pre-existing pressure overload - induced heart failure	Administration of BH4 recouples the uncoupled eNOS and reverses pre-existing heart failure	2
	Cardiopulmonary bypass (dog)	Application of BH4 improves myocardial, endothelial and pulmonary function after cardiopulmonary bypass with hypothermic cardiac arrest	55

Table 2. Overview of relevant cardiovascular clinical trials with BH4-analogs.

Cardiovascular disease	Dosage of BH4	Outcome	Reference
Hypertension	Oral 5 mg/kg, twice daily, eight-weeks	Not statistically significant drop of 6.4 mm Hg in patients' SBP	2008, BioMarin Pharmaceutical NCT00325962
Pulmonary Arterial Hypertension	2.5 mg/kg/day for two weeks, 5 mg/kg/day for two weeks, 10 mg/kg/day for four weeks, then 20 mg/kg/day for two days	n/a	Ongoing Vanderbilt University, The National Institutes of Health, GCRC& BioMarin Pharmaceutical NCT00435331
Endothelial Dysfunction	5 mg/kg oral, BID 13.5 days	Not significant.	2009, BioMarin Pharmaceutical NCT00532844
Sickle Cell Disease	16-week dose escalation phase oral, every 4 weeks as follows: 2.5, 5, 10(once-daily), and 20 mg/kg/day(twice-daily), continue in an optional extension phase at the highest tolerated dose for up to a total of 2 years	Improvement of endothelial function	2009, BioMarin Pharmaceutical NCT00445978
Peripheral Arterial Disease	400 mg oral, BID 24 weeks	Not significant.	2009, BioMarin Pharmaceutical NCT00403494

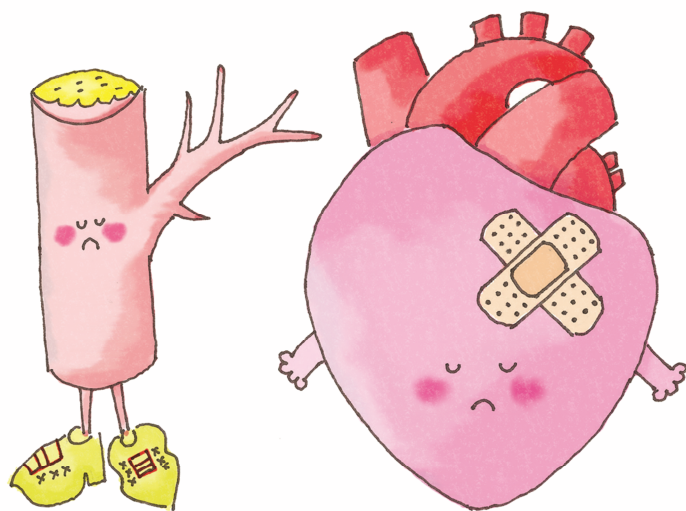
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Chapter 3

Tackling endothelial dysfunction by modulating NOS uncoupling: new insights into its pathogenesis and therapeutic possibilities

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Abstract

Endothelial nitric oxide synthase (eNOS) serves as a critical enzyme in maintaining vascular pressure by producing nitric oxide (NO); hence, it has a crucial role in the regulation of endothelial function. The bioavailability of eNOS-derived NO is crucial for this function and might be affected at multiple levels. Uncoupling of eNOS, with subsequently less NO and more superoxide generation, is one of the major underlying causes of endothelial dysfunction found in atherosclerosis, diabetes, hypertension, cigarette smoking, hyperhomocysteinemia, and ischemia/reperfusion injury. Therefore, modulating eNOS uncoupling by stabilizing eNOS activity, enhancing its substrate, cofactors, and transcription, and reversing uncoupled eNOS are attractive therapeutic approaches to improve endothelial function. This review provides an extensive overview of the important role of eNOS uncoupling in the pathogenesis of endothelial dysfunction and the potential therapeutic interventions to modulate eNOS for tackling endothelial dysfunction.

Introduction

The endothelial layer of blood vessels is critical to vascular and myocardial health and plays an important role in the pathophysiology of hypertension and myocardial ischemia. It regulates the release of 1) endothelium-induced relaxing factors such as nitric oxide (NO) and endothelium-derived hyperpolarization factor (EDHF), 2) endothelium-derived contracting factors such as endothelin-1 and angiotensin, and 3) proinflammatory prothrombotic and 4) growth factors. Endothelial dysfunction is characterized by an impaired release of the aforementioned and has been defined as a blunting of the vasodilatory response to acetylcholine or hyperemia, both of which are known to produce NO-dependent vasodilatation. Disruption of the endothelium triggers a number of signaling cascades that converge on medial smooth muscle cells, which stimulates cell proliferation and migration and leads to pathological repair and development of neointimal hyperplasia ¹. Endothelial dysfunction causes NO deficiency ², which has been implicated in the underlying pathology of many cardiovascular diseases.

Endothelial nitric oxide synthase (eNOS) is the critical enzyme in the maintenance of vascular pressure by producing NO, a volatile gas that diffuses to the adjacent vascular smooth muscle. NO has a physiological role in the regulation of vascular tone, synaptic transmission, and cellular defense. In addition, it plays a major role in the relaxation of smooth muscle surrounding the arterioles ³ and maintaining vascular function by inhibition of vasoconstriction ⁴, platelet aggregation, leukocyte adhesion, and cell proliferation through the cGMP-dependent downstream signaling cascade. Moreover, NO limits oxidative phosphorylation in mitochondria ⁵. NO functions not only as a physiological regulator of cell respiration but also augments the generation of mitochondria-derived reactive oxygen species (ROS). The majority of ROS generation in the vasculature is derived from NADPH oxidases (NOX) and eNOS uncoupling. The latter takes place when oxidative stress oxidizes the fragile eNOS cofactor tetrahydrobiopterin (BH4).

This review gives an extensive overview of the role of eNOS uncoupling in the pathogenesis of endothelial dysfunction and how modulating eNOS uncoupling can tackle endothelial dysfunction.

Molecular Mechanism of eNOS Uncoupling

Evidence has shown that eNOS uncoupling is the underlying cause of endothelial dysfunction in animal experiments such as deoxycorticosterone acetate (DOCA)-salt hypertension ⁶, angiotensin II-induced hypertension ⁷, myocardial ischemia/reperfusion (I/R) injury ⁸, streptozotocin (STZ)-induced diabetes ⁹, as well as

essential hypertension¹⁰ and hypertension-induced heart failure¹¹. Importantly, uncoupling of eNOS in the pathogenesis of endothelial dysfunction in vascular disease states has been linked to the decrease of BH4 bioavailability due to enhanced oxidation of BH4. As an essential cofactor, BH4 is necessary for optimal eNOS activity¹². It facilitates NADPH-derived electron transferring from the eNOS reductase to the oxygenase domain to convert L-arginine to NO and L-citrulline. When BH4 levels are inadequate, eNOS becomes unstable and uncoupled, leading to subsequently less NO production and more superoxide generation. Moreover, the interaction between NO and superoxide leads to the formation of peroxynitrite, a potent oxidant, which further oxidizes BH4¹³.

BH4 is synthesized by the de novo or the salvage pathway. Through de novo pathway, BH4 is generated from guanosine-5'-triphosphate (GTP) via the rate-limiting enzyme GTP cyclohydrolase (GTPCH), 6-pyruvoyltetrahydropterin synthase, and sepiapterin reductase^{14, 15}. In the salvage pathway, BH4 is regenerated by its oxidized form 7,8-dihydrobiopterin (BH2) via dihydrofolate reductase (DHFR) or quinonoid dihydrobiopterin (qBH2) through dihydropteridine reductase (DHPR)^{15, 16}. Interestingly, BH2 can promote eNOS uncoupling because BH2, which has no eNOS cofactor property, can competitively replace eNOS-bound BH4¹⁷. In addition, the relative abundance of eNOS vs. BH4, together with the intracellular BH4:BH2 ratio, rather than absolute concentrations of BH4, is the key determinant of eNOS uncoupling¹⁸. Recently, Crabtree et al.¹⁹ demonstrated in BH4 deficiency hph-1 mice that DHFR plays a vital role in regulating the BH4:BH2 ratio and eNOS coupling in vivo, particularly when total biopterin availability is diminished.

The bioavailability of NO produced by eNOS might be affected at multiple levels, including 1) eNOS mRNA or protein expression, 2) availability of its substrate L-arginine, which might be competed by asymmetric dimethylarginine (ADMA), 3) availability of its cofactors, 4) protein-protein interaction such as caveolin-1 (cav-1) and heat shock protein 90 (Hsp90), 5) posttranslational modifications, and 6) reaction of NO with superoxide to yield peroxynitrite, which further reduce the bioavailability of BH4²⁰. Although many studies have shown that eNOS overexpression generates vasoprotective effects by increasing endothelium-derived NO^{21,22}, the expression of eNOS protein in endothelial dysfunction remains at normal or even, in some cases, increased levels³. Indeed, elevated eNOS expression without further increase in BH4 levels, results in eNOS uncoupling because of the enzyme cofactor imbalance²³ (see Figure 1).

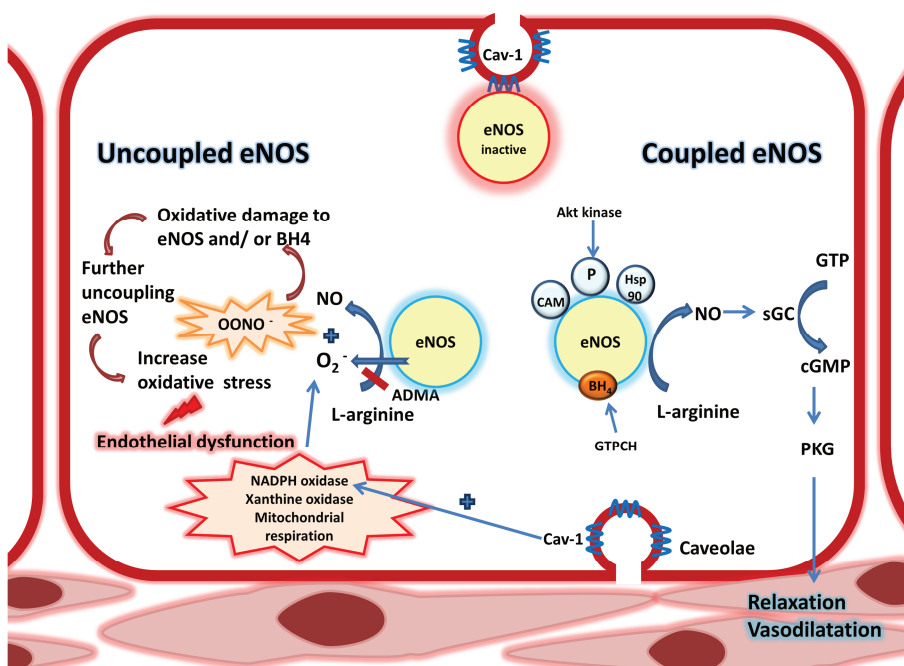


Figure 1. Central role of endothelial NO synthase (eNOS) uncoupling in the pathogenesis of endothelial dysfunction.

eNOS is localized at the plasma membrane caveolae. In endothelial cells, eNOS is inactive when it is bonded with caveolin 1 (cav-1). When it becomes active, eNOS disassociates from cav-1 and binds with calmodulin (CAM) and heat shock protein 90 (Hsp90) and together with phosphorylation of serine sites (e.g., Ser1177). The functional eNOS protein is a dimer (so-called coupled eNOS). Tetrahydrobiopterin (BH4), an essential cofactor of eNOS, is necessary for optimal eNOS activity. BH4 facilitates NADPH-derived electron transferring from the eNOS reductase to the oxygenase domain to convert L-arginine to NO and L-citrulline. NO plays a major role in relaxation of smooth muscle surrounding arterioles and maintaining vascular function by inhibition of vasoconstriction, platelet aggregation, leukocyte adhesion, and cell proliferation through the cGMP-dependent downstream signaling cascade. Interaction between L-arginine and asymmetric dimethylarginine (ADMA; endogenous competitive inhibitor of NOS) is likely direct competition for eNOS. When availability of L-arginine or BH4 levels are inadequate, eNOS becomes unstable and uncoupled, leading to subsequently less NO production and more superoxide generation. Moreover, interaction between NO and superoxide leads to formation of peroxynitrite, a potent oxidant, which further oxidizes BH4, resulting in eNOS uncoupling as a vicious cycle, with subsequent endothelial dysfunction. Outside arrows indicate adjacent endothelial cells.

The availability of the substrate L-arginine is required as the nitrogen donor for eNOS-derived NO. Although it is unlikely that plasma L-arginine levels would drop below the concentrations required for eNOS activity, decreased intracellular L-arginine caused by arginase may also lead to eNOS uncoupling. The expression of arginase in endothelial cells (EC) can compete with eNOS for their common substrate²⁴ and downregulate eNOS activity²⁵. Furthermore, studies show that oxidized low-density lipoprotein (oxLDL) is able to reduce endothelial L-arginine uptake, resulting in decreased local L-arginine and eNOS uncoupling²⁶. In addition, endogenous ADMA levels, a derivative of L-arginine, can act to competitively inhibit eNOS. The elevated levels of plasma ADMA are associated with oxidative stress within the vascular and development of endothelial dysfunction and cardiovascular diseases^{27, 28}.

Atherosclerosis-Induced eNOS Uncoupling

Endothelial dysfunction is considered to be an early marker for atherosclerosis, preceding angiographic or ultrasonic evidence of atherosclerotic plaque formation²⁹. Atherosclerosis starts with an innate immune response involving the recruitment and activation of monocytes/macrophages that respond to an excessive accumulation of modified lipids within the arterial wall, followed by an adaptive immune response³⁰. Wilcox et al.³¹ demonstrated that atherosclerosis is associated with enhanced expression of all forms of NOS in the intima and adventitia. Furthermore, NOS-derived NO plays a dual role, as both anti- and proatherosclerotic effects are based on the course of disease progression.

Endothelium-dependent vascular relaxation is impaired both in cholesterol-fed mice³² and in isolated human coronary arteries³³, and this impairment is correlated with the degree of atherosclerosis³⁴. Administration of L-arginine^{35, 36} or BH4^{37, 38} attenuates atherosclerotic lesion progression, whereas administration of NOS inhibitors blocks this protective effect³⁹, signifying a direct link between NO and atherosclerosis lesion formation. Recently, using an apoE^{-/-} model of rapid atherosclerotic lesion formation after partial carotid ligation, Li et al.⁴⁰ demonstrated that NOS uncoupling and BH4 deficiency contribute to the vascular inflammation and abnormal cytokine milieu induced by disturbed flow without affecting systemic immune cell numbers.

In apoE^{-/-} mice, aortic superoxide production is diminished after administration of the NOS inhibitor L-NAME, suggesting that NOS is an important source of superoxide in this model^{41, 42}. In addition, BH4 deficiency is likely the major cause of eNOS uncoupling in atherosclerosis. apoE^{-/-} mice overexpressing eNOS (apoE^{-/-}/eNOS-Tg) showed eNOS dysfunction demonstrated by a decrease in NO production relative to eNOS expression, a marked reduction in vascular BH4, and an increase in superoxide formation, resulting in an acceleration of atherosclerotic lesion

formation⁴³. In addition, oral BH4 administration ($10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) to apoE^{-/-}/eNOS-Tg mice reduced the formation of atherosclerotic lesion and vascular superoxide and increased eNOS-derived NO⁴³, indicating that the increase in eNOS expression alone, but not together with vascular BH4 levels, may result in eNOS uncoupling due to the stoichiometric relationships between endothelial BH4 and NOS activity²³. On the other hand, overexpression of GTPCH in apoE^{-/-}/GTPCH-Tg mice is able to decrease endothelial superoxide production, increase aortic BH4 levels, and recouple eNOS. As a result, these double transgenic mice showed a significant decrease in aortic root atherosclerotic plaque compared with apoE^{-/-} controls⁴¹. Takaya et al.⁴⁴ demonstrated that, in the triple transgenic mice of eNOS- and GTPCH-overexpressing in apoE^{-/-} (apoE^{-/-}/eNOS-Tg/GTPCH-Tg), increases in vascular BH4 levels are associated with decreased eNOS-dependent superoxide production and results in reduced atherosclerotic plaque area. This study indicates that eNOS-derived superoxide plays a crucial role in atherosclerosis and that upregulation of GTPCH is able to restore eNOS function.

Elevated levels of C-reactive protein (CRP), a proinflammatory marker, have been also associated with atherosclerosis. Many data are evolving to suggest that CRP also promotes atherothrombosis^{45, 46}. Interestingly, studies in human endothelial cells showed that CRP, a proinflammatory marker, caused downregulation of eNOS activity and NO bioavailability^{47, 48}. Moreover, expression of human CRP in transgenic mice resulted in decreased eNOS activity⁴⁹⁻⁵¹. Singh et al.⁵² demonstrated in human aortic endothelial cells (HAEC) that CRP inhibited GTPCH and stimulated NOX, leading to a decrease in BH4 and an increase in ROS levels. These resulted in uncoupling of eNOS and decreased eNOS activity, which was associated with decreased phosphorylation of Ser¹¹⁷⁷ and decreased eNOS binding to Hsp90.

In healthy vasculatures, eNOS-derived NO prevents oxidative modification of low-density lipoprotein (LDL)⁵³. However, under oxidative stress, LDL is initially oxidized at the endothelial walls^{54, 55}. oxLDL has been known to initiate the pathogenesis of atherosclerosis^{56, 57}. Moreover, oxLDL is also a potent inducer of superoxide; hence, it is a cause of oxidative stress⁵⁸. oxLDL increases synthesis of caveolin-1 (cav-1)⁵⁹ and inhibits the Akt survival pathways⁶⁰⁻⁶² attenuating eNOS activity with subsequent decreases in NO bioavailability.

During the inflammatory process in atherosclerosis, inducible NOS (iNOS) in endothelial cells and macrophages is upregulated and produces excessive amount of NO⁶³. Deletion of iNOS decreases plaque development in apoE^{-/-} mice, indicating that iNOS does have proatherogenic effects⁶⁴. In addition, studies indicate that neuronal NOS (nNOS) also plays a role in protecting atherosclerotic plaque development in animal models^{65, 66}. This NOS isoform is upregulated in smooth

muscle cells, macrophages, and endothelial cells in both early and advanced human atherosclerotic lesions³¹. Recently, Seddon et al.⁶⁷ demonstrated in healthy humans that nNOS-derived NO is important for the control of blood pressure via regulation of basal vascular tone and blood flow, yet there are no data to indicate nNOS uncoupling is involved with atherosclerosis.

Diabetes Mellitus-Induced eNOS Uncoupling

Evidence indicates that endothelial dysfunction could play an initial and ultimately crucial role in the development of macrovascular and microvascular complications caused by diabetes mellitus (DM) in human and animal models of DM⁶⁸. Under hyperglycemic conditions, a hallmark of diabetes, eNOS-derived NO capacity is diminished⁶⁹. In vitro experiments have shown that protein kinase C (PKC)-mediated phosphorylation of eNOS protein may elicit the enzyme catalytic activity⁷⁰. Stimulation of endothelial cells with phorbol esters, direct activators of PKC⁷¹, or glucose⁷² elevates the expression of eNOS. Glucose also greatly enhances endothelial superoxide production⁷², leading to increased vascular formation of the NO/superoxide reaction product peroxynitrite⁷³, which further promotes BH4 oxidation, with subsequent enhanced eNOS uncoupling⁷⁴.

Insulin is an essential hormone of metabolic homeostasis that has vasodilator action via PI3K/Akt pathway-dependent eNOS activation⁷⁵. In addition to the PI3K/Akt pathway, insulin can modulate eNOS activity by increasing BH4 synthesis via activation of GTPCH. Shinozaki et al.⁷⁶ have demonstrated that the GTPCH activity is significantly decreased in a rat model of insulin resistance, which leads to attenuation of endothelial BH4 levels and substantial increased BH2 levels, resulting in impairment of endothelium-dependent vasodilatation. Alteration of insulin-mediated vasodilation has been associated with vascular insulin resistance, which is implicated in arterial hypertension and endothelial dysfunction. eNOS-derived NO regulates blood flow to insulin-sensitive tissues, including skeletal muscle⁷⁷, liver⁷⁸, and adipose tissue⁷⁷, and its activity is impaired in insulin-resistant individuals. An increasing body of evidence has shown the role of eNOS in regulating tissue sensitivity to insulin. An increase in systemic insulin resistance has been demonstrated in eNOS^{-/-} mice that received a hyperinsulinemic euglycemic clamp^{79, 80}. Furthermore, a study in normotensive and nondiabetic individuals demonstrated that insulin resistance, measured by insulin-mediated glucose disposal, significantly correlated with increased plasma levels of ADMA, a potent endogenous NOS inhibitor⁸¹. It was been demonstrated that NOS inhibition impaired microvascular recruitment and blunted muscle glucose uptake in response to insulin⁸² indicating that eNOS plays a major role in the regulation of insulin sensitivity. Interestingly, an increase in eNOS expression is not always beneficial. It was demonstrated in Goto-Kakizaki rats,

a model of insulin resistance, that elevated eNOS expression in aortic tissue was associated with increased superoxide production and decreased NO bioavailability. Incubation with L-NAME or deendothelialization of the aortic segments significantly decreased superoxide production.

Increased superoxide production in diabetes is not restricted to EC and is demonstrably enhanced in the smooth muscle layer⁸³. Interestingly, adenoviral transfection of eNOS to diabetic vessels improved endothelial-dependent relaxation without altering superoxide production of vascular smooth muscle cells, an observation that may point to an important contribution of an impaired NOS function to endothelial dysfunction in diabetes.

Importantly, downregulation of DHFR has been observed in aortas of STZ-induced diabetic mice⁸⁴. As a key enzyme responsible for salvaging BH4, DHFR downregulation can result in BH4 deficiency and BH2 accumulation, both of which worsen eNOS uncoupling. Crabtree et al.¹⁷ has demonstrated in hyperglycemic models of murine endothelial cells that eNOS uncoupling is due to the accumulation of BH2 in these cells. In addition, BH2 replaces BH4-eNOS binding and, hence, directly suppresses eNOS activity. This indicates that BH₂-eNOS assembly does have a key role in the pathogenesis of diabetic vasculopathies. In STZ-diabetic rats, hyperglycemia-induced oxidative stress impairs the ability of dimethylarginine dimethylaminohydrolase (DDAH) to metabolize ADMA, leading to an elevation of ADMA and inhibition of endothelium-derived synthesis of NO⁸⁵.

Leo et al.⁸⁶ demonstrated in diabetic rats that hyperglycemia increased superoxide production, causing endothelial dysfunction, which was due to the impairment of both NO and EDHF-type relaxation. However, the degree of impairment in NO activity appears to be greater than the reduction in EDHF activity, as EDHF is able to elicit maximum relaxation when NO is inhibited, suggesting that NO is more susceptible to impairment by diabetes. Recent studies showed that attenuation of eNOS-derived NO in insulin-resistant C57BL/KsJ (diabetic, *+db/+db*) mice⁸⁷ and rat models of type 1 and type 2 DM⁸⁸ could be due to an impairment of eNOS activity through the augmented cav-1 expression, which further indicates that cav-1 plays an important role in cardiovascular complications in both types of diabetes.

Recently, Jo et al.⁸⁹ demonstrated in STZ-induced diabetic cardiomyopathy in eNOS^{-/-}, iNOS^{-/-}, nNOS^{-/-}, and WT mice that iNOS uncoupling plays a major role in the pathophysiology of the diabetic heart. Although the oxidative/nitrosative stress markers, i.e., malondialdehyde (MDA), 4-hydroxynoneal (HNE), and nitrotyrosine (NT) are augmented in the diabetic mouse heart, this increase in oxidative/nitrosative stress was significantly repressed in the iNOS^{-/-} diabetic mouse heart. Importantly, oral administration of sepiapterin, a precursor of BH4 (10 mg·kg⁻¹·day⁻¹ for 14 days)

significantly increases myocardial BH4 in the control and diabetic hearts. However, a significant increase in the ratio BH4:BH2 is observed only in the diabetic heart and is associated with the inhibition of uncoupling NOS, resulting in increasing iNOS-derived NO. Intriguingly, the absence of the increase in BH2 in the diabetic heart by administration of sepiapterin suggests that the salvage pathway of BH4 synthesis through DHFR is more activated in the diabetic heart than in the control heart. These findings may provide an important therapeutic implication in the treatment of NOS uncoupling-induced cardiovascular dysfunction by potentiating the salvage pathway of BH4 synthesis by sepiapterin administration. Furthermore, hyperglycemia significantly elicits the expression of iNOS both in *in vitro*^{90, 91} and *in vivo* diabetic embryopathy mouse models associated with increased protein nitrosylation and enhanced NO and superoxide production, resulting in excess production of peroxynitrite, which stimulates the apoptosis pathway via c-Jun NH₂-terminal kinase1/2 (JNK1/2) activation⁹¹. Presently, no evidence demonstrates uncoupling of nNOS in DM.

Hypertension-Induced eNOS Uncoupling

It is broadly recognized that ROS contribute to the pathogenesis of hypertension. Increased levels of superoxide oxidize NO to peroxynitrite and subsequently to nitrite and nitrate. This results in a loss of “bioactive” NO-mediated vasodilatation, an increase in vasoconstriction, and subsequently an increase in systemic vascular resistance⁹². Angiotensin II is known to play a major role in the initiation and progression of hypertension⁹³. It stimulates hypertension in part by ROS generation via eNOS uncoupling, leading to further increases in superoxide^{7, 16}. Furthermore, the reduction of NO caused by eNOS uncoupling promotes salt sensitivity and salt-induced hypertension⁹⁴. Landmesser et al.⁶ demonstrated in DOCA salt hypertensive rats that uncoupled eNOS and NOX represent important sources of increased vascular ROS production that ultimately oxidize vascular BH4. In addition, Takimoto et al.¹¹ and Moens et al.⁹⁵ revealed in a mouse model of transverse aortic constriction that eNOS uncoupling is a major source of superoxide in the pathogenesis of de novo and established pressure overload-induced heart failure. Compared with WT, eNOS^{-/-} mice showed no cardiac hypertrophy, dilatation, and myocardial fibrosis because there was no detrimental eNOS-derived superoxide production.

Higashi et al.¹⁰ demonstrated that BH4 augments endothelium-dependent vasodilatation in the forearm in normotensive as well as hypertensive individuals through the recoupling of eNOS and by increasing eNOS-derived NO production. Moreover, studies indicate that the use of NOS inhibitors with relative specificity for nNOS and iNOS⁹⁶⁻⁹⁸ implicates reduced NO production by these two NOS isoforms as possible contributors to salt-induced hypertension. Indeed, nNOS-derived NO

generation in autonomic efferent nerves participates in vasodilatation, blood flow increase, and hypotension^{99, 100}. Silberman et al.¹⁰¹ demonstrated in a mild hypertensive mouse model (unilateral nephrectomy, with subcutaneous implantation of DOCA and saline feeding) increased cardiac oxidation, decreased NOS-derived NO generation and decreased cardiac BH4, resulting in NOS uncoupling. Interestingly, experiments of NOS inhibitors in this study revealed that nNOS is the largest contributor to cardiac superoxide production in diastolic dysfunction.

Smoking-Induced eNOS Uncoupling

Cigarette smoke (CS)-mediated oxidative stress downregulates eNOS levels, leading to reduced NO production and decreased endothelium-dependent vasodilatation in endothelial cells^{102, 103}. Edirisinghe et al.¹⁰² revealed that CS-mediated downregulation of vascular endothelial growth factor receptor 2 expression and activation results in reduction of phosphorylated eNOS and total eNOS both in human lung microvascular endothelial cells and in mouse lungs. Decreased expression/activation levels of eNOS in response to CS have direct implications for endothelial functions such as cell migration, angiogenesis, and endothelium-dependent relaxation¹⁰⁴. Importantly, deficit of BH4 bioavailability caused by ROS scavengers in CS may lead to NOS uncoupling, at least in part, contributing to endothelial dysfunction in chronic smokers¹⁰⁵. In addition, CS has been shown to induce iNOS expression in pulmonary arteries¹⁰⁶. Furthermore, Lowe et al.¹⁰⁷ revealed that chemicals found in aqueous cigarette smoke extracts can directly affect eNOS by oxidizing BH4. Although both eNOS and nNOS are inhibited by cigarette smoke extracts, BH4 reactivates eNOS but not nNOS, whereas L-arginine protects nNOS but not eNOS. Thus, differential effects might be expected, and this may be the basis for isoform-selective inhibition of nNOS over eNOS in penile tissue from rats treated with cigarette smoke¹⁰⁸.

Homocysteine-Induced eNOS Uncoupling

Increased plasma homocysteine (Hcy) has emerged as an independent risk factor for atherosclerosis and vascular disease¹⁰⁹. The pathophysiological roles of hyperhomocysteinemia (HHcy) in endothelial dysfunction are associated with increased thrombogenicity^{110, 111}, increased oxidative stress¹¹², overactivation of redox-sensitive inflammatory pathways¹¹³, and atherogenesis¹¹⁴. Uncoupling of eNOS is mainly responsible for Hcy-induced oxidative stress, because Hcy activates intracellular superoxide synthesis in human umbilical vein endothelial cells (HUVEC), and treatment with L-NAME markedly decreases superoxide generation, indicating that Hcy-induced oxidative stress is dependent mainly on NOS activity¹¹⁵. In cultured porcine endothelial cells incubated with Hcy, it has been described that eNOS uncoupling contributes to Hcy-induced superoxide formation¹¹⁶. The interaction

between Hcy and eNOS uncoupling can be explained in several ways. First, Hcy has been shown to decrease L-arginine uptake in endothelial cell cultures, with a subsequent uncoupling of eNOS¹¹⁷. Second, Hcy elicits the cellular transport of L-arginine without altering eNOS activity, and eNOS generates superoxide rather than NO and forms peroxynitrite which later oxidizes BH4, leading to eNOS uncoupling¹¹⁵. Third, studies with HUVEC have revealed that Hcy induces eNOS uncoupling through increasing superoxide and diminishing intracellular BH4 bioavailability^{115, 118}. Recently, He et al.¹¹⁹ provided the first evidence that Hcy impairs coronary artery endothelial function. In addition, plasma levels of NO and BH4 are positively correlated and significantly decreased in patients with HHcy compared with controls. This suggests that the uncoupling of eNOS induced by HHcy in patients with chronic HHcy due to reduced BH4 levels may explain in part this adverse effect¹²⁰. Fourth, Hcy inhibits catabolic degradation activity of DDAH, causing ADMA accumulation; thus, it inhibits eNOS activity in vascular endothelium¹²¹. Importantly, ADMA is associated with the increased eNOS uncoupling found in endothelium of patients with coronary artery disease (CAD)¹²². Recently, Lemarie et al.¹²³ demonstrated in the endothelial progenitor cells (EPC) of heterozygous methylenetetrahydrofolate reductase-deficient (*Mthfr*^{+/-}) mice, which are mildly HHcy, and that ROS production is significantly increased and the eNOS dimer-to-monomer ratio significantly decreased compared with EPC from wild-type mice, demonstrating eNOS uncoupling. This study indicates that eNOS uncoupling is a main cause of increased ROS formation in these mildly HHcy mice. Interestingly, the expression of sirtuin-1 (SIRT1), a NAD⁺-dependent protein deacetylase, is impaired at both mRNA and protein levels by *Mthfr*^{+/-} mice. SIRT1 has been shown to exert protective effects against endothelial dysfunction by preventing stress-induced senescence¹²⁴ and, at vascular levels, to promote endothelium-dependent vasodilation by deacetylating eNOS and increasing NO bioavailability¹²⁵. These results suggest that, in mildly HHcy mice, the uncoupling of eNOS increases ROS production, which leads to inhibition of SIRT1, then to premature senescence of EPC, and thus eventually to endothelial dysfunction.

On the other hand, although there are no data showing that Hcy induces nNOS or iNOS uncoupling, Hcy has also been shown to upregulate iNOS, which may contribute to the inflammatory response that characterizes atherogenesis and may account for the adverse effects of Hcy¹²⁶. In addition, an increase in iNOS activity is a key contributor to a HHcy-mediated collagen/elastin switch and a resulting decline in aortic compliance¹²⁷.

I/R Injury-Induced eNOS Uncoupling

One of the events occurring in the early phase of I/R is vascular dysfunction associated with impaired endothelial function. ROS production has been implicated in

endothelial damage, leading to endothelial dysfunction after I/R injury¹²⁸. ROS generation has been shown to be significantly increased during reperfusion. The largest increase in ROS level has been observed 15 min after reperfusion¹²⁹⁻¹³¹. This increase in reactive oxygen burst coincides with the development of endothelial dysfunction. Indeed, while 90 or 120 min of ischemia did not change vascular reactivity, vascular response to acetylcholine (ACh) was attenuated as early as 2.5 min after the onset of reperfusion^{132, 133}. A reduction in endothelial-dependent vasorelaxation after I/R injury has been associated with a decrease in NO bioavailability due to alteration of eNOS. Furthermore, endothelial-dependent vasorelaxation in response to serotonin was also impaired in pig carotid artery exposed to I/R, which was again improved with BH4 supplementation¹³⁴.

Several ROS generators during I/R injury have been determined. These include mitochondria¹³⁵, xanthine oxidase¹³⁶, NOX¹³⁷, neutrophils¹³⁸, and eNOS¹³⁹. eNOS uncoupling has been demonstrated to have a significant role in the occurrence of tissue damage after I/R injury. In the early phase of cardiac ischemia, there is an increase in Ca^{2+} uptake by cardiomyocytes and endothelial cells, leading to activation of eNOS with subsequent rapid and short-lived aggravation of NO concentration. This is followed not only by rapid consumption of L-arginine (the substrate of the enzymes) but also BH4 (the cofactor), triggering eNOS uncoupling and a decrease in NO bioavailability, which has been shown especially in the onset of reperfusion^{140, 141}.

It has been demonstrated that isolated rat hearts subjected to ischemia showed reduced BH4 levels with subsequent decreased eNOS activity and increased superoxide production¹⁴². BH4 supplementation could restore impaired epicardial coronary epithelial function; moreover, it attenuated lipid peroxidation and improved cardiac functional recovery in rat models of global cardiac I/R¹⁴³. In accordance with that, impaired coronary flow during reperfusion phase could be improved by BH4 infusion, which was associated with increased eNOS activity and decreased NOS-dependent superoxide production¹⁴². Moreover, a study in rat femoral artery also showed that BH4 increased NO production and decreased H_2O_2 release after I/R.

In addition to BH4, Hsp90, another posttranslational modulator of eNOS, has also been implicated in I/R injury. Hsp90 was proposed to prevent eNOS uncoupling and eNOS-derived superoxide production after chronic myocardial ischemia¹⁴⁴. It has been demonstrated that an Hsp90-transfected pig model of myocardial I/R showed reduction in infarct size and improved myocardial function. This effect was abrogated by administration of L-NAME and was associated with the ability of Hsp90 to act as an adaptor for Akt and phosphatase calcineurin, thereby promoting eNOS Ser¹¹⁷⁷ phosphorylation and Thr⁴⁹⁵ dephosphorylation¹⁴⁵. All these findings enhance

the essential role of eNOS in I/R injury and provide evidence for the potential of this protein as a therapeutic target.

Atrial Fibrillation-Induced eNOS Uncoupling

The association of eNOS and atrial fibrillation (AF) has been derived from the study of Minamino et al.¹⁴⁶, showing significantly reduced plasma levels of nitrite and nitrate and platelet cGMP in patients with AF compared with patients with sinus rhythm. Furthermore, Takahashi et al.¹⁴⁷ demonstrated that, compared with control patients with sinus rhythm, the increase of ACh-induced forearm blood flow was considerably smaller in patients with AF and was improved after cardioversion to sinus rhythm. These two studies proposed a correlation between AF and endothelial dysfunction with eNOS as a link. Altered hemodynamic condition has been implicated as an underlying mechanism that translates AF to endothelial dysfunction^{147, 148}. Cai et al.¹⁴⁸ suggested that decreased endocardial eNOS expression in a dog model of AF was due to turbulent blood flow occurring during AF. In an in vitro setting, it was demonstrated that HUVEC produce lower levels of NO when exposed to turbulent flow compared with when in laminar flow conditions. An increase in NO generation after laminar flow exposure appeared to be due to an upregulation of eNOS expression¹⁴⁹. Regulation of eNOS expression and NO generation by shear stress involves the opening of ion channels followed by eNOS protein phosphorylation and gene upregulation, leading to an increase in NO bioavailability¹⁵⁰. Although experimental animal studies have shown reduced eNOS expression in fibrillating atria^{148, 151}, a human study showed no difference in eNOS gene and protein expression in the atrium of AF and sinus rhythm (SR) patients¹⁵².

In addition to eNOS, iNOS has been implicated in AF. Han et al.¹⁵³ reported an increased level of NO in the right atrium of AF patients, with subsequent generation of peroxynitrite, which was associated with an increase in iNOS expression. In addition, Nishijima et al.¹⁵⁴ reported increased expression of iNOS in a dog model of heart failure-induced AF. Despite some concerns about the methodology used^{155, 156}, the authors demonstrated that increased expression of iNOS in heart failure (HF) is accompanied by low NO and high superoxide production, which can be reversed by administration of BH4, suggesting the occurrence of iNOS uncoupling. Furthermore, 6-wk oral administration of 50 mg BH4 plus 3 g L-arginine twice daily reduced inducible AF and normalized heart failure-induced shortening of the left atrial myocyte action potential duration in this model¹⁵⁴.

Investigating the source of superoxide in the atrium of patients with AF, Kim et al.¹⁵⁷ demonstrated that gp91phox containing NOX in atrial myocytes was the main source of atrial superoxide production in SR and in AF patients. NOS was shown to contribute significantly to superoxide generation in fibrillating atria but not in patients

with sinus rhythm, suggesting that increased levels of NOX-dependent oxidative stress induce eNOS uncoupling with a consequence of increased production of superoxide. Moreover, Reilly et al.¹⁵⁸ demonstrated that different atrial sources of ROS varied with the duration and substrate of AF. The authors reported that NOX was the main source of superoxide production in the left atrium of goats after 2 wk of AF and in patients who developed postoperative AF. Conversely, after 6 mo of AF, NOX showed no production of superoxide. Instead, NOS was the main source of oxidative stress in the presence of this long-standing AF. Interestingly, NOS-dependent superoxide production was associated with decreased BH4 level and BH4:BH2 ratio, suggesting that NOX-dependent superoxide generation during early periods of AF induces oxidation of BH4 as a cofactor of NOS leading to uncoupling of this enzyme. These results imply that AF treatments may be differentiated according to duration and substrate of AF. Whether agents that modulate eNOS will be able to alter AF in patients with atrial structural remodeling remain to be elucidated.

Endothelial Dysfunction and Endothelial Progenitor Cells

EPC are circulating cells with the ability to differentiate into mature endothelium and have a role in endothelial repair and maintenance. Under certain pathophysiological conditions, for instance in CAD patients, this process seems to be blunted, resulting in reduced levels and migratory capacity of EPC for neovascularization of ischemic tissue¹⁵⁹. In addition, both NO and oxidative stress, and in particular the balance between them, regulate the number and function of EPC by direct and indirect mechanisms¹⁶⁰. Increasing evidence suggests that a damaged endothelial lining can be restored by circulating EPC derived from bone marrow¹⁶¹⁻¹⁶³. A decrease in the number and function of EPC has been associated with a large number of risk factors for atherosclerosis^{164, 165}. There is strong evidence that EPC-expressed eNOS is regulated under various physiological and pathophysiological conditions. Moreover, compounds or molecules that increase eNOS expression improve EPC function, whereas eNOS inhibitory substances have deleterious effects¹⁶⁶. By upregulating eNOS expression of EPC, statins, estrogen, and erythropoietin (EPO) could enhance reendothelialization or augment neovascularization¹⁶⁷⁻¹⁷⁰. On the contrary, oxLDL or CRP attenuates EPCs' survival, differentiation, and function by reducing eNOS expression of EPC^{171, 172}.

It has been demonstrated that several substances affect the number and function of EPC via an Akt/eNOS-related pathway. Indeed, the PI3K/Akt pathway is vital for regulating EPC recruitment, mobilization, and proliferation¹⁷³. Compounds that stimulate the PI3K/Akt protein kinase pathway can also activate eNOS⁶². This association between eNOS and EPC count and activity appears to be crucial, because the expression of eNOS is essential for the mobilization of stem and progenitor cells

¹⁷⁴, and perturbations in the PI3K/Akt/eNOS/NO signaling pathway or one of its members may result in EPC dysfunction ¹⁵⁶. Recently, Cui et al. ¹ demonstrated that transplantation of EPC overexpressing eNOS can repair balloon-caused carotid artery injury in a rat model by inhibiting neointimal hyperplasia and restoring vascular function.

Diagnosis of Endothelial Dysfunction

The ability to detect endothelial dysfunction before overt cardiovascular disease manifests makes the diagnostic modalities attractive clinical tools for prevention and rehabilitation ¹⁷⁵. In humans, endothelium-derived vasodilatation can be assessed by measuring increases in the diameter of large arteries (forearm or coronary) after 1) release of ischemia (e.g., arrested forearm circulation), 2) intra-arterial infusion of ACh, or 3) a sudden blood pressure elevation by placing the hands in ice water (cold pressure test). These measurements are based on the fact that conduit vessels can respond to alterations in blood flow by increasing vessel diameter via an endothelial-dependent mechanism ^{176, 177}. These responses have been shown to reflect local bioactivity of endothelium-dependent NO generation and NO inactivation. These measurements are very sensitive to external conditions (room temperature, caffeine intake, etc.); hence, these measurements need to be performed under standard conditions ¹⁷⁸.

Quantitative coronary angiography (QCA) measures the changes in the diameters of the coronary arteries in baseline conditions compared with vasodilatation induced by endothelium-dependent drugs. More recently, noninvasive QCA has been developed using computed tomography imaging ¹⁷⁹ or magnetic resonance imaging (MRI) ¹⁸⁰.

Therapeutic Effects of eNOS Modulators

L-arginine

L-arginine, a semiessential amino acid found in large quantities in fish, chicken, and beans, is the substrate for the production of NO. L-arginine deficiency or the presence of its endogenous inhibitors, i.e., ADMA, may lead to eNOS uncoupling ¹⁸¹. L-arginine activates oxygen uptake by eNOS ¹⁸² and prevents superoxide generation within uncoupled eNOS by electron interaction with heme-bound oxygen ¹⁸³. In addition, L-arginine may be able to restore the physiological status by normalizing the extracellular L-arginine:ADMA ratio ¹⁸⁴. Although L-arginine improves both endothelium-dependent vasodilation and abnormal interactions of vascular cells, platelets, and monocytes, clinical studies with L-arginine have shown inconsistent effects on endothelial function. Acute and subacute L-arginine administration improves

NO-dependent vasodilatation in a study of healthy elderly individuals¹⁸⁵ and patients with hypercholesterolemia¹⁸⁶ and coronary artery disease¹⁸⁷. On the other hand, chronic administration may not show the beneficial effect of L-arginine. The VINTAGE MI study demonstrated that 6 mo of oral L-arginine administration (3 g 3 times a day) does not improve clinical outcome and possibly increases risk of death in older patients with CAD¹⁸⁸. In addition, L-arginine supplementation also leads to an increase in Hcy production, which can result in worsening, not improving, endothelial function and atherosclerosis¹⁸⁹. Oral administration of L-arginine (9 g/day) to healthy postmenopausal women for 1 month has no effect on major endocrine hormones or lipid profile. Although L-arginine could be the key to future treatment of cardiovascular disorders, it has not been possible to draw any general conclusion supporting the use of L-arginine for improving the clinical treatment in patients with endothelial dysfunction. In addition, it is not completely clear which types of endothelial dysfunction are related to L-arginine deficiency.

BH4

Since uncoupled eNOS can increase the production of ROS, promote BH4 oxidation, and self-limit its NO biosynthesis, modulating eNOS uncoupling is an attractive therapeutic approach in endothelial dysfunction. The most straightforward way to modulate eNOS is administration of its essential cofactor BH4¹²⁰.

BH4 is an FDA-approved therapy for some forms of phenylketonuria (PKU), in which there is a deficiency in the hepatic enzyme phenylalanine hydroxylase. Supplementing with BH4 increases NOS activity by recoupling uncoupled eNOS in mice with hypertension-induced heart failure with subsequent reversal of cardiac hypertrophy and fibrosis⁹⁵. *In vitro* administration of BH4, or its physiological precursor sepiapterin, restored endothelium-dependent relaxation of resistance arteries in diabetic *db/db* mice¹⁹⁰, atherosclerotic vessels of humans and pigs³⁸, and patients with endothelial dysfunction^{105, 191}. Chronic oral administration of BH4 has been reported to improve endothelium function^{6, 192}, decrease vascular superoxide production, and increase vascular NO production. As a result, it blunts the increase in blood pressure in a hypertensive mice model⁶. Recently, Li et al.⁴⁰ demonstrated in apoE^{-/-} mice by using partial carotid ligation that oral BH4 supplementation in drinking water (10 mg·kg⁻¹·day⁻¹ for 1 wk) prevents NOS uncoupling and improves endothelial function associated with diminished monocyte adhesion and T-cell activation as well as blunted cytokine production from the vessel wall. In addition, short-term administration of BH4 (intra-arterial infusion 500 µg/min) improves endothelial-dependent vasodilatation to ACh in type 2 diabetes¹⁰⁵. Porkert et al.¹⁹³ demonstrated in subjects with poorly controlled hypertension that orally administering (400 mg or higher) BH4 daily has a significant and sustained antihypertensive effect. Importantly,

this effect is associated with reversing the uncoupling of NOS and improving NO bioavailability⁸⁰. Although studies suggest that BH4 administration shows beneficial results for endothelial dysfunction, it may have limited long-term benefit in improving eNOS coupling¹⁷. Chronic BH4 administration may result in endothelial BH2 accumulation and consequent eNOS uncoupling because BH2 can replace eNOS-bound BH4 competitively^{17, 194}. Recently, Moens et al.¹⁹⁵ studied the therapeutic efficacy of BH4 in hypertension-induced heart failure in a mouse model of pressure overload, demonstrating a bimodal dose-dependent relationship, first rising and then declining at higher doses. Importantly, this study indicates that, at higher doses of exogenous BH4 (and thus BH2), the intrinsic capacity to maintain BH4:BH2 ratios may become compromised, limiting net efficacy. These results expose a potential limitation for the clinical use of BH4 in diseases characterized by oxidative stress, as defining the oral dose that would optimally provide BH4 rather than BH2 may be difficult and vary from individual to individual. Inadequate conversion of BH2 to BH4 may pose a limitation to its therapeutic use, and coadministration of agents that stimulate such conversion may be valuable to enhance its therapeutic benefits in vivo. Albeit, the benefit of a BH4 supplement, i.e., synthetic sapropterin hydrochloride (6R-BH₄, Kuvan) on endothelial function seems promising, the results from clinical trials were disappointing, not because they revealed adverse effects but for their lack of efficacy. Future studies are needed to determine the dose-response relation between BH₄ and its effect on superoxide generation in models of cardiovascular diseases because of the tight stoichiometric relation between eNOS and BH₄, with possible subsequent uncoupling.

Folic acid

Folic acid (FA) is a chemically stable and inexpensive vitamin (Vit B9), which has direct and indirect superoxide scavenging effects. Although the best-known biological function of FA is to reduce plasma Hcy, the major effects of FA in the cardiovascular system are independent of Hcy lowering¹⁹⁶. Moat et al.¹⁹⁷ demonstrated in patients with CAD that FA dose-dependently improves endothelial function through a mechanism independent of Hcy lowering. Clinically, FA and 5-methyltetrahydrofolate (5-MTHF), the active form of FA, have been shown to restore endothelial function in patients with hypercholesterolemia¹⁹⁸, diabetes^{199, 200}, and atherosclerosis²⁰¹. FA increases the vascular bioavailability of BH4 and subsequently reduces eNOS-derived superoxide generation²⁰¹. FA restores the function of uncoupled eNOS by improving BH4 bioavailability in the vasculature by preventing its oxidation, enhancing the regeneration of BH4 from BH2, and chemically stabilizing BH4^{95, 202}. Furthermore, Hyndman et al.²⁰³ demonstrated that 5-MTHF is capable of binding the active site of NOS and mimicking the orientation of BH4. In addition, van Etten et al.¹⁹⁹ demonstrated that administration of 5-MTHF ameliorates endothelial

dysfunction found in patients with type 2 diabetes by restoring impaired NO-mediated vasodilatation. Moreover, administration of FA improves EPC function by normalizing gene expression profiles in type 1 diabetes patients²⁰⁰. However, recent clinical trials have failed to demonstrate a benefit of long-term use of FA in lowering the risk of recurrent cardiovascular disease or death after an acute myocardial infarction^{204, 205}, which can be explained by too low of a dosage²⁰⁶. Therefore, an important distinction must be made between FA as a long-term, low-dose fortification or dietary supplement and as a short-term, high-dose treatment.

eNOS transcription enhancers

The novel small molecules AVE9488 and AVE3085 are eNOS transcription enhancers. AVE9488 has proven *in vivo* effects improving left ventricular remodeling in a rat model of myocardial infarction²⁰⁷ and a mouse model of cardiac I/R injury²⁰⁸. Wohlfart et al.⁴² demonstrated in a model of experimental atherosclerosis that both AVE9488 and AVE3085 have vasoprotective properties, i.e., the increased endothelial NO generation associated with reduced cuff-induced neointima formation and reduced formation of atherosclerotic plaques in apoE^{-/-} mice. This study indicates that both compounds showed the concomitant increase in eNOS transcription and eNOS protein levels; BH4 levels also increased, although GTPCH mRNA levels did not. In addition, the mechanisms for the increase in BH4 by these eNOS enhancers still remain unknown. Recently, Yang et al.²⁰⁹ demonstrated that AVE3085 restored impaired endothelial function in a hypertensive model by upregulated expression of eNOS protein and mRNA, enhanced eNOS phosphorylation, and decreased formation of nitrotyrosine. While these preclinical data are very promising, no clinical studies are initiated at the moment.

Statins

Statins, hydroxymethylglutaryl-CoA reductase inhibitors, improve endothelial function by increasing eNOS stability²¹⁰ and bioavailability of NO²¹¹, and in part by lowering LDL²¹². In animal experiments, statins have been shown to reduce platelet activation and thrombus formation, which in part, are influenced by eNOS upregulation²¹³. Moreover, statins increase BH4 levels in vascular endothelial cells by potentiating GTPCH gene expression and BH4 synthesis, thereby preventing relative shortages of BH4²¹⁴. Some statins, such as atorvastatin, decrease cav-1 expression in EC, thereby allowing for the activation of eNOS by cofactors, resulting in promoting NO production²¹⁵. Wenzel et al.²¹⁶ demonstrated in STZ diabetic rats that chronic administration of atorvastatin (20 mg·kg⁻¹·day⁻¹ for 7 wk) improves endothelial function by normalizing endothelial BH4 level and GTPCH expression, reducing oxidative stress, preventing NOX upregulation, and preventing and reversing eNOS uncoupling.

Conclusions

In this review, we have discussed how eNOS uncoupling is one of the major underlying causes of endothelial dysfunction found in atherosclerosis, diabetes, hypertension, cigarette smoking, HHcy, and I/R injury. Evidence suggests that modulating of eNOS by stabilizing eNOS function and suppressing eNOS-derived ROS is a promising therapeutic target for endothelial dysfunction. Therapeutics, such as L-arginine, BH4, FA, eNOS transcription enhancers (AVE9488 and AVE 3085), and statins have achieved vascular protection, improving endothelial function and ameliorating cardiovascular diseases in animal and/or human studies (Figure 2), yet this experimental evidence needs to be confirmed in clinical trials. Further understanding of the pathophysiology and the molecular biology of endothelial dysfunction is required to understand this discrepancy. Importantly, future efforts may be directed at identifying signaling pathways regulating NOS on oxidative/nitrosative stress and providing possible mechanisms that can recouple the uncoupled eNOS and other NOS isoforms. These new studies will provide us with a comprehensive understanding of the molecular basis of eNOS uncoupling, thereby identifying potential novel therapeutic approaches targeting the underlying signaling pathways for the prevention and treatment of progressive endothelial dysfunction in cardiovascular diseases.

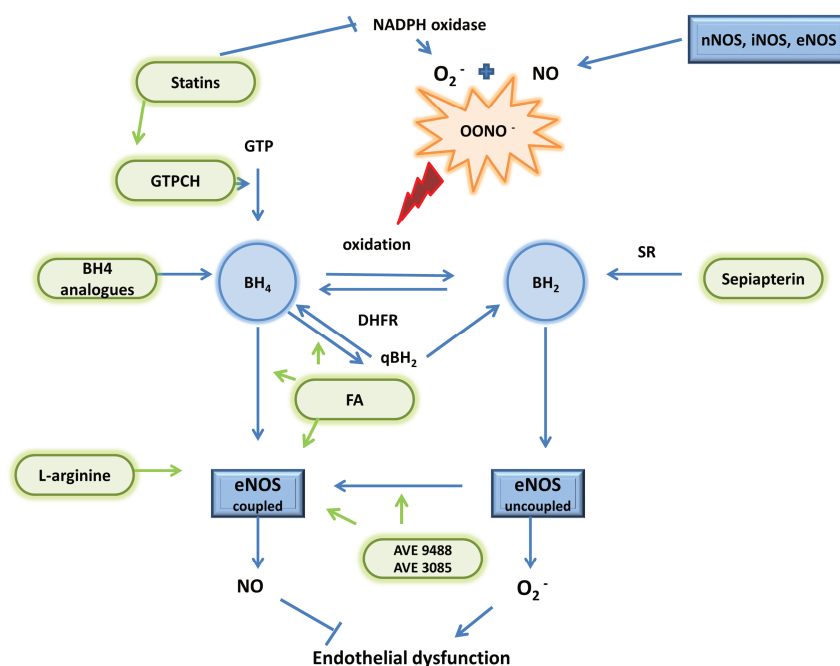


Figure 2. Potential therapeutic options for treating endothelial dysfunction by modulating eNOS.

The most straightforward way to modulate eNOS is administration of its substrate L-arginine or its essential cofactor BH₄ or BH₄ analogs. Folic acid (FA) can modulate eNOS by improving BH₄ bioavailability in the vasculature by preventing its oxidation, enhancing regeneration of BH₄ from BH₂, and chemically stabilizing BH₄. Novel small molecules AVE9488 and AVE3085 are eNOS transcription enhancers. Statins can improve endothelial function by increasing eNOS stability and inhibiting NADPH oxidase upregulation. Also, statins can increase BH₄ levels in vascular endothelial cells by potentiating GTPCH gene expression and BH₄ synthesis.

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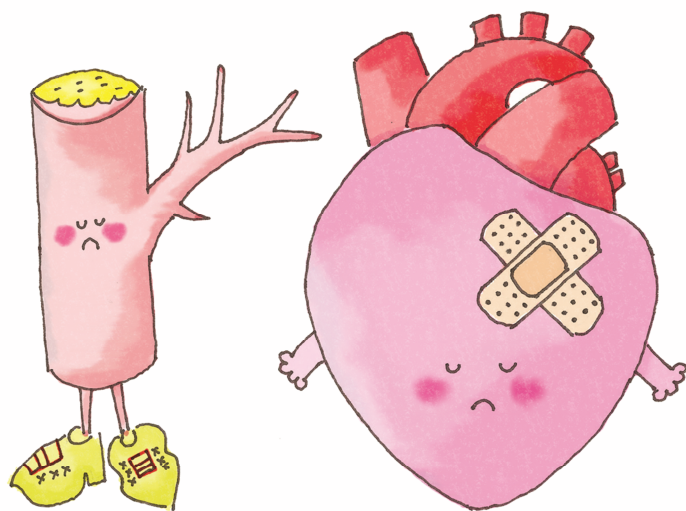
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Chapter 4

Enhancing eNOS transcription: a new therapeutic option to tackle myocardial and endothelial dysfunction

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(In preparation)

Abstract

Nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS) has antithrombotic, antiatherosclerotic, antihypertensive, and antihypertrophic effects under physiological conditions. Normal function of eNOS requires dimerization of the enzyme and the presence of its cofactor tetrahydrobiopterin (BH₄). A functional eNOS oxidizes its substrate L-arginine to L-citrulline and NO. As a consequence of eNOS uncoupling, eNOS generates superoxide rather than NO, contributing to the pathogenesis of cardiovascular diseases. The development of novel pharmacological approaches targeting eNOS is challenging, especially the search for compounds that have multiple, simultaneous actions: 1) increase eNOS expression and/or activity, 2) augment its cofactor BH₄ and 3) reverse eNOS uncoupling. Importantly, compounds that increase eNOS protein levels are only beneficial if eNOS functionality is guaranteed. AVE9488 and AVE3085 are novel small molecule compounds that transcriptionally enhance eNOS gene expression, which are aimed to increase signaling NO and bioavailability. In this review we have outlined the mechanisms and therapeutic benefits of these novel eNOS transcription enhancers in cardiovascular disease.

1 Introduction

Nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS) has antithrombotic, antiatherogenic, and antihypertensive properties¹. Moreover, eNOS-dependent NO has antihypertrophic effects via downstream NO-cyclic guanosine monophosphate (GMP)-protein kinase G (PKG) signaling cascades². Impairment of NO bioavailability leads to altered regulation of key physiological and cellular processes, such as vasodilatation, platelet function, angiogenesis, apoptosis and smooth muscle cell proliferation. Hence, it plays a crucial role in the pathogenesis of cardiovascular diseases, hallmarked by endothelial dysfunction and/or arterial or cardiac hyperplasia³. Inadequate eNOS-dependent NO bioavailability may result from: 1) a decrease in eNOS expression and/or its activity, 2) deficiency in eNOS substrate (L-arginine) or its cofactor (tetrahydrobiopterin; BH4), 3) the presence of endogenous inhibitors (asymmetric dimethylarginine; ADMA), and 5) eNOS uncoupling and accelerated NO degradation by reactive oxygen species (ROS) (see reviews^{3,4}). Therefore, therapeutic enhancement of NO bioavailability holds promise as cardiovascular-protective strategy.

A straightforward method to increase NO bioavailability are exogenous NO donors, such as nitroglycerin and sodium nitroprusside. However, this approach is limited by drug tolerance development after long-term treatment and toxicity issues. This indicates that alternative strategies are required⁵. One such alternative are agents that modulate endogenous NO bioactivity, such as angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers, statins, phosphodiesterase inhibitors and β -blockers⁶. These agents are primarily targeting distinct processes, but have been reported to indirectly increase eNOS gene expression, eNOS activity or to stabilize eNOS mRNA⁷⁻¹¹.

A more direct approach that is widely explored are inducers of eNOS protein expression. However, these only are beneficial if the ensuing eNOS protein is functional¹², in other words able to oxidize its substrate L-arginine to L-citrulline and NO¹³. Appropriate eNOS function requires dimerization of the enzyme and abundant presence of its cofactor tetrahydrobiopterin (BH4). This renders the development of novel drugs that induce eNOS activity challenging. This particularly holds when aiming at compounds that display multiple eNOS enhancing functions, and simultaneously 1) increase eNOS expression and/or activity, 2) augment bioavailability of its cofactor BH4, and 3) recouple eNOS. Screening of compound libraries for eNOS transcriptional upregulation yielded two structurally related indan-based small-molecular-weight compounds: AVE9488 (4-fluoro-*N*-indan-2-yl-benzamide; CAS no. 291756-32-6; empirical formula C₁₆H₁₄FNO; earlier designation C2431) and AVE3085 (2,2-difluoro-benzo[1,3]dioxole-5-carboxylic acid indan-2-ylamide; CAS no. 450348-

85-3; empirical formula $C_{17}H_{13}F_2NO_3$). These small molecule compounds display a high potency for transcriptional enhancement of eNOS gene expression, and could potentially increase signaling NO and bioavailability^{14, 15}. In this review we shall outline the mechanisms and therapeutic benefits of these novel eNOS transcription enhancers in cardiovascular diseases.

2 AVE mechanism of action

2.1 eNOS transcription-dependent effects of AVE

The mode of action of AVE 9488 and AVE 3085 capacity to enhance eNOS expression has been explored *in vitro* in a human endothelial cell line (EA.hy 926) and in human umbilical vein endothelial cells (HUVEC). Both compounds enhanced eNOS promoter activity in a concentration-dependent manner, without affecting eNOS mRNA stability¹⁵. Thus, AVE compounds seem to act at eNOS transcriptional level¹⁵. Silencing of transcription factors Sp1, GATA2 and PEA3, which are critical in controlling eNOS promoter activity, resulted in decreased basal activity of eNOS promoter, but did not impact eNOS transcription activity¹⁵. Thus, the responsible transcription factors/cis-elements still remain to be identified. The efficacy of AVE9488 to increase eNOS expression was comparable to that of simvastatin¹⁵, which was seen to elevate eNOS stability by prolonging half-life of eNOS mRNA, but had little effect on eNOS transcription⁹.

2.2 eNOS activity-dependent effects of AVE

A potential secondary mode of action of AVE compounds involves direct regulation of eNOS activity. Phosphorylation of serine (Ser)¹¹⁷⁷ (corresponding to Ser¹¹⁷⁶ in rodents) is associated with increased eNOS activity, whereas phosphorylation at threonine (Thr)⁴⁹⁵ inhibits eNOS activity¹⁶⁻¹⁹. In homocysteine induced endothelial dysfunction, AVE3085 increased eNOS phosphorylation at Ser¹¹⁷⁷ in a phosphoinositide (PI) 3 kinase-protein kinase B (Akt)-dependent manner²⁰. AVE 3085 increased eNOS activity in reoxygenation after cardioplegia-induced hypoxia²¹ and in ADMA-induced endothelial dysfunction²² in porcine coronary small arteries studies. This was achieved through enhanced phosphorylation of eNOS (p-eNOS) at Ser¹¹⁷⁷^{21, 22} and decreasing Thr⁴⁹⁵ phosphorylation²². Conversely, AVE 3085 treatment did not alter the ratio of phosphorylation at Thr⁴⁹⁵ or Ser¹¹⁷⁶ to total eNOS in aortas of diabetic mice, indicating that AVE3085 did not affect eNOS activity²³. These discrepancies may be due to p-eNOS quantification methods i.e. p-eNOS/ glyceraldehyde 3-phosphate dehydrogenase (GAPDH) *versus* p-eNOS/total eNOS. The latter study suggested that the increase in phosphorylated eNOS mainly reflected a proportional increase in eNOS synthesis upon AVE treatment²³.

2.3 eNOS transcription-independent effects of AVE

2.3.1 eNOS uncoupling and BH4 bioavailability

Next to altering eNOS transcription and activity, AVE9488 and AVE3085 were also shown to reverse eNOS uncoupling^{15, 20, 22}. Although this notion has been challenged for AVE3085, where the increased eNOS activity resulted in increased NO production. However, this was accompanied by an overproduction of peroxynitrite in diabetic CD34+ stem cells²⁴. This further suggested that NADPH oxidase, but not eNOS uncoupling, was a major source of ROS generation in this study, and thus increasing eNOS expression without correcting oxidative stress was not effective.

Uncoupling of eNOS activity may in part be ascribed to AVE's effect on BH4 availability. Treatment of AVE9488 in a model of cuff-induced neointima formation in mice resulted in increased vascular BH4 levels, without altering the expression of the rate limiting enzyme in *de novo* synthesis of BH4, i.e. guanosine triphosphate cyclohydrolase I (GTPCH)¹⁵. In contrast, AVE9488 treatment did not alter myocardial BH4 levels in a cardiac ischemia/reperfusion (I/R) mouse model²⁵. Of note, AVE9488 increased vascular BH4 contents when administered *in vivo*, but not in endothelial cells *in vitro*, suggesting that the elevation of BH4 *in vivo* may be indirect, e.g. by AVE9488 effects on cell types other than endothelial cells¹⁵.

It is unclear how AVE9488 exactly induced intravascular BH4 levels. It did not protect from ROS-induced BH4 oxidation though regulation of superoxide-generating NADPH oxidases (NOX), including NOX1-4 and p22phox and superoxide-scavenging enzymes, i.e. superoxide dismutase1-3 (SOD)^{15, 25}. In contrast, AVE3085 may protect oxidative degradation of BH4 by ROS through downregulation of NOX subunits p22phox and gp91phox²⁶ and iNOS gene expression^{20, 26}, suggesting AVE3085 reduced ROS generation through regulation of these oxidant enzymes. Moreover, both AVE compounds did not have superoxide and peroxynitrite scavenging activity by themselves^{23, 25}.

2.3.2 Arginase activity

AVE may also act indirectly by influencing substrate availability. eNOS activity can be affected by the endogenous inhibitor ADMA, and indirectly by the activity of arginase, which competes with eNOS for L-arginine as substrate. Induction of expression level or activity of arginase I or ADMA will compromise substrate levels and thus NO production at the expense of a rise in superoxide generation^{27, 28}. It has been demonstrated in homocysteine (Hcy)-induced endothelial dysfunction that AVE3085 reversed iNOS upregulation and arginase activation^{20, 27}. However, by what

mechanisms AVE3085 inhibits Hcy-induced iNOS upregulation remains to be elucidated.

2.4 eNOS-dependent effector pathways in AVE therapeutic activity

2.4.1 Regulation of endothelial dysfunction

AVE 3085 was reported to improve endothelial function in both conduit²⁹ and resistance vessels²³, and has blood pressure lowering properties²⁹. This suggested that improved endothelial function might be related to its anti-hypertensive effect, indicating a cardiovascular protective effect. Moreover, AVE 9488 and AVE3085 have shown to protect against cardiac and vascular remodeling through downregulation of TGF- β , connective tissue growth factor (CTGF)²⁶, A-type and B-type natriuretic peptide (ANP and BNP, respectively)³⁰, b- major histocompatibility complex (b-MHC) expression, as well as mothers against decapentaplegic (Smad) 2/3 signaling³¹. In addition, bone marrow-derived mononuclear cells from patients with ischemic cardiomyopathy (ICMP) have increased NO/cGMP/PKG signaling, which is accompanied by impaired stromal cell-derived factor 1 (SDF-1) dependent migratory capacity. SDF-1 migratory responses could be normalized by AVE9488 treatment¹⁴. In contrast, AVE3085 did not improve CD34+ cell migration in response to SDF-1 α in diabetics³².

2.4.2 Vasodilator-stimulated phosphoprotein (VASP)

NO-dependent phosphorylation of VASP is an essential regulatory step in VASP-dependent inhibition of platelet activation. Therefore, VASP phosphorylation is positively correlated with NO bioavailability, and can thus be used as a sensitive (albeit indirect) measure of defective NO/cGMP signaling³³. Treatment with AVE9488 increased platelet VASP- phosphorylation at Ser¹⁵⁷ and Ser²³⁹ in I/R mice²⁵, myocardial infarction (MI) rats³⁰ and severe congestive heart failure rats³⁴, indicating that AVE9488 exerts inhibitory effects on platelet activation.

2.4.3 Mobilization of progenitor cells

Cell therapy with bone marrow-derived stem/progenitor cells is a novel therapeutic strategy for improving neovascularization and cardiac function in peripheral arterial disease, ischemic heart disease, and ICMP³⁵⁻³⁷, which has been associated with eNOS/NO function. Indeed, the expression of eNOS is vital for the mobilization of stem and progenitor cells³⁸. Moreover, increased eNOS expression of endothelial progenitor cells (EPC) by statins, estrogen or erythropoietin improved EPC function, was associated with enhanced re-endothelialization or augmented neovascularization³⁹⁻⁴².

Sasaki et al.¹⁴ reported that *ex vivo* pretreatment with AVE9488 (5 μ M for 18-24h) of bone marrow-derived mononuclear cells (BMC) derived from patients with ICMP resulted in increased eNOS mRNA expression in peripheral blood-derived EPC, CD34⁺ cells and BMC as well as increased NO production in lineage-negative CD105⁺ bone marrow-derived cells (i.e. circulating endothelial progenitor cells). Moreover, AVE-pretreated BMC from patients restored the impaired stromal cell-derived factor 1 (SDF-1) mediated migratory capacity and this effect was abolished following administration of antibodies directed against the SDF-1 receptor, C-X-C chemokine receptor type 4 (CXCR4), or L-NAME. In addition, AVE 9488 did not improve the impairment of SDF-1 mediated migratory capacity in BMC derived from either eNOS deficient mice or cGMP-dependent protein kinase I deficient mice (PKG1^{-/-}, also termed GKI^{-/-}). This indicated that AVE9488 effects were mediated by eNOS, and increased NO/cGMP/PKG signaling activity was associated with the improvement of SDF-1 mediated migratory capacity.

Intravenous infusion of BMC derived from ICMP patients in mouse model of unilateral hind limb ischemia *in vivo* resulted in decreased recovery of limb perfusion after induction of hind limb ischemia, highlighting the importance of stem cell quality and functional activity. AVE-pretreated BMC derived from ICMP patients enhanced recovery of limb perfusion and increased neovascularization, associated with increased physical activity as assessed by swimming test. In addition, injection with AVE-treated patient-derived BMC in a mouse model of MI resulted in improvement of ejection fraction and stroke work. In summary, AVE9488 increased eNOS expression enhances the regenerative capacity of EPC isolated from ICMP patients suggesting a synergistic effect between cell and gene therapy.

In conclusion, both AVE compounds have largely similar cardioprotective effects by reversing eNOS uncoupling, without affecting superoxide and peroxynitrite scavenging activity. In addition, they impact on BH4 bioavailability and arginase activity, as well as the expression of oxidant enzymes and superoxide inducers, while eNOS activity upon AVE 3085 or AVE9488 treatment is diverging or opposite. The broad spectrum of cardioprotective activities of the AVE compounds are summarized in Figure1.

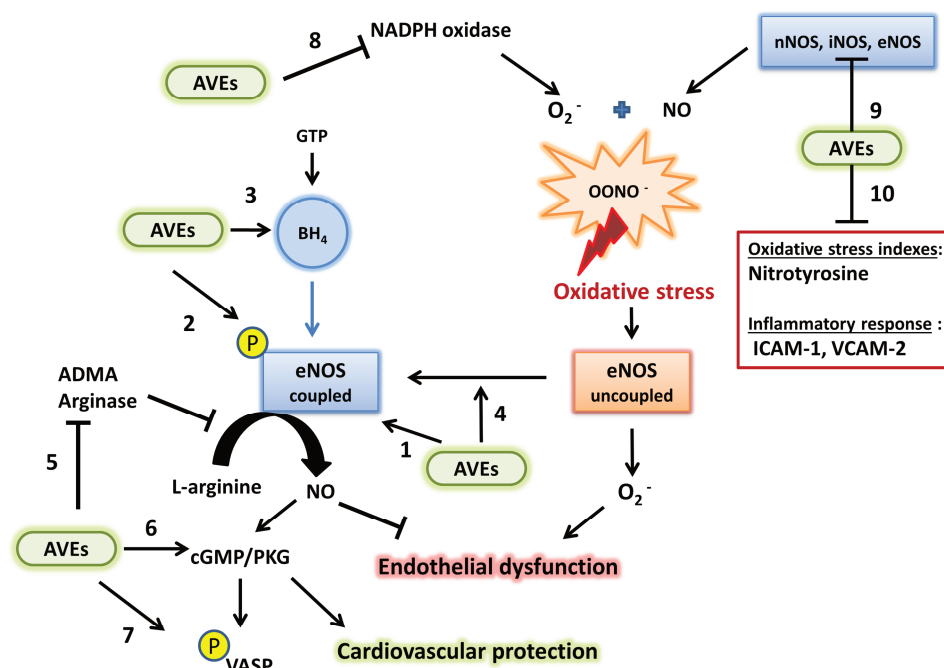


Figure 1. The potential mechanisms for the cardiovascular protective effect of AVE9488 and AVE3085, the eNOS transcription enhancers.

AVE compounds (AVEs) increase eNOS mRNA and protein expression (1), increase eNOS catalytic activity (by promoting phosphorylation of eNOS (p-eNOS) at Ser1177 and decreasing of p-eNOS at Thr495 (2) and by increasing BH₄ bioavailability (3), and in addition can reverse eNOS uncoupling amongst others (4). Finally, AVEs promote eNOS mediated NO production indirectly by downregulating arginase and ADMA activity, (5). In turn, the elevated NO production will inhibit endothelial dysfunction, and provide cardiovascular protection via cGMP-dependent PKG signaling pathway (6). In parallel, NO can increase phosphorylation of vasodilator-stimulated phosphoprotein (VASP), platelet activation (7). Finally, AVEs decrease superoxide generation through downregulation of NADPH oxidase (8) and iNOS expression (9), and decrease oxidative stress and inflammatory responses (10). Abbreviations explained in main text.

3 Preclinical studies of AVE's cardiovascular protective activity

3.1 Hypertension and associated endothelial dysfunction

Loss of NO production and/ or bioavailability results in increased vasoconstriction, and subsequently increased systemic vascular resistance and hypertension⁴³. AVE compounds has shown to increased eNOS expression and activity, and subsequently increased NO generated by eNOS^{15, 23}. Targeting endogenous eNOS production instead of directly relaxing vascular smooth muscle may represent an alternative therapeutic approach to the traditional treatment strategy

by preventing the pathological changes in endothelial cells in hypertension. Yang et al.²⁹ demonstrated that treatment of AVE3085 (10mg/kg/day, 4 wks) in spontaneously hypertensive rats (SHR) resulted in reduced systolic blood pressure, restored the impaired endothelium-dependent relaxations SHR aortae *in vitro*, and increased aortic eNOS mRNA and protein expression, and phosphorylation. In addition, AVE3085 blunted the increased formation of nitrotyrosine, a marker of NO-dependent oxidative stress, in SHR rats. This suggests that AVE3085 might exert anti-hypertensive effects, possibly through increased eNOS expression and activity, and inhibited vascular oxidative stress.

3.2 Diabetes mellitus

Evidence showed that eNOS uncoupling was associated with diabetes mellitus (DM), causing reduced NO bioavailability and increased superoxide production⁴⁴. Under hyperglycemic conditions, eNOS-derived NO capacity was diminished⁴⁵. Moreover, studies suggested that the re-endothelialization capacity of EPC derived from diabetic patients, including the migration capacity in response to SDF-1 and vascular endothelial growth factor (VEGF), were severely impaired⁴⁶⁻⁴⁸.

Jarajapu et al.²⁴ demonstrated that diabetic CD34+ cells exhibited impairment of migratory response to SDF-1 α . Treatment with AVE3085 (10 μ M, 24h) in diabetic CD34+ cells resulted in increased eNOS mRNA expression without improving migration in response to SDF-1 α *in vitro*, while improving CD34+ cells homing functions in a mouse model of retinal I/R injury *in vivo*. Interestingly, diabetic CD34+ cells treated with AVE3085 displayed increased peroxynitrite generation, which was further increased following SDF-1 α stimulation. Pretreatment with peroxynitrite scavenger ebselen abolished this increase, suggesting that the AVE3085-mediated effect was dependent on overproduction of peroxynitrite. In addition, diabetic CD34+ cells displayed increased NOX2 mRNA expression, increased NADPH oxidase activity, as well as increased superoxide generation. Co-incubation with L-NAME did not alter superoxide levels, suggesting that superoxide generation was not caused by eNOS uncoupling. Importantly, treatment with NOX inhibitors improved vasoreparative functions in the dysfunctional diabetic CD34+ cells *in vitro* and *in vivo*, resulting in enhanced NO bioavailability. Taken together, this suggested that NOX2 was a major source of superoxide generation over uncoupled eNOS in this particular diabetes study, and targeting NOX activity might thus be suitable for diabetes treatment. While these findings are intriguing, it is difficult to draw a conclusion on the role of eNOS-generated superoxide in diabetic EPC due to inadequate details of patient characteristics. Indeed, the differences in stages and/or severity of diabetes, and antidiabetic treatments such as insulin, have shown different outcomes of the sources of superoxide generation⁴⁹. Activation of NOX likely occurs

early in diabetes and/or in mild diabetic forms⁵⁰, whereas eNOS uncoupling with an even stronger subsequent production of ROS predominantly exists in advanced diabetes⁵¹.

Cheang et al.²³ demonstrated that co-treatment with AVE3085 (1 $\mu\text{mol/L}$) restored the reduction of endothelium-dependent relaxation (EDR) to acetylcholine (ACh) in aortas of diabetic db/db and in aortas of wild-type (WT) mice exposed to high glucose. This effect was abolished either by co-incubation with the transcription inhibitor actinomycin D, by L-NAME as well as in eNOS deficient mouse aortas, suggesting that AVE 3085-induced improvement of vascular relaxation was mediated by eNOS and its transcription process. In addition, treatment with AVE 3085 (1 $\mu\text{mol/L}$) reversed the reduction of NO bioavailability the primary endothelial cells from mouse aortas-exposed caused by high glucose. Moreover, co-treatment with AVE3085 (1 $\mu\text{mol/L}$) reversed eNOS downregulation, and inhibited ROS generation in high glucose-exposed WT aortas, as well as in diabetic mouse aortas. Interesting, high glucose-exposed WT aortas exhibited eNOS uncoupling, assessed by a decrease in eNOS dimer to monomer ratio and treatment with AVE3085 remained unaffected. In addition, AVE3085 treatment was unaffected in attenuation of the increased aortic ROS generation induced by hypoxanthine and xanthine oxidase in WT mice, suggesting that AVE3085 did not have a direct ROS-scavenging effect. In an *in vivo* study, oral administration of AVE3085 (10 mg/kg/day for 7 days) restored endothelial dysfunction in diabetic mouse arteries and inhibited aortic superoxide generation. While AVE3085 treatment increased aortic eNOS protein expression, it did not alter the ratio of phosphorylation at Thr⁴⁹⁵ or Ser¹¹⁷⁶ to total eNOS in diabetic mice. This suggested that the effect of AVE3085 to reduce ROS was unlikely mediated through inhibiting eNOS uncoupling, but by increasing eNOS expression and endothelial-derived NO generation.

3.3 Atherosclerosis

Endothelial dysfunction is generally considered to be a key process in atherogenesis, and concordant with this notion, atherosclerotic lesions have been shown to exhibit decreased eNOS mRNA and protein expression and eNOS enzymatic dysfunction^{43, 52, 53}. This suggests that normalizing eNOS function might be beneficial in atherosclerosis. Adenoviral overexpression of eNOS, but not iNOS, in a rabbit model of atherosclerosis resulted in improved endothelial NO-mediated vasorelaxation in atherosclerotic arteries, and blunted plaque progression⁵⁴. In contrast, apolipoprotein deficient (apoE^{-/-}) atherosclerotic mice overexpressing eNOS (apoE^{-/-}/eNOS-Tg) displayed markedly reduced aortic BH4 levels as well as increased eNOS uncoupling, leading to accelerated plaque formation. Such statements raise a note of caution concerning the aimed level of increase in eNOS

expression, without concordant increases in vascular BH4 levels. This may result in eNOS uncoupling due to the stoichiometric relationships between endothelial BH4 and NOS activity⁵⁵ (Table 1).

AVE may perform better in that regard. Wohlfart et al.¹⁵ demonstrated that sub-chronic administration of AVE 9488 (30mg/kg/day) for 17 days to WT mice significantly increased eNOS protein expression in healthy aortas (50%) and femoral arteries (48%). AVE9488 treatment (10 mg/kg/day, gavage b.i.d. for 17 days) in cuff-induced neointima formation in apoE^{-/-} mice resulted in increased aortic eNOS protein expression and decreased neointima formation. Similarly, chronic administration of AVE9488 or AVE3085 (30 mg/kg/day) for 12wks in apoE^{-/-} mice-fed Western diet resulted in decreased plaque formation and increased eNOS protein expression without altering plasma lipid profiles or heart rate. Importantly, AVE compounds were ineffective in inhibiting neointima formation and atherosclerosis in eNOS^{-/-} mice or apoE^{-/-}/eNOS^{-/-} mice, respectively, indicating that the effects of AVE compounds were strictly eNOS-dependent. In addition, untreated atherosclerotic apoE^{-/-} mice exhibited increased aortic ROS production compared to AVE9488-treated group. This was partly inhibited by NOS inhibitor L-NAME suggesting that ROS was partly produced by an uncoupled eNOS. In contrast, AVE9488-treated apoE^{-/-} mice (30 mg/kg/day, 2wks) displayed increased aortic BH4 levels. Also, AVE9488 reduced aortic ROS production to levels that could not be lowered any further by L-NAME, indicating effective recoupling of eNOS. Notably, the increase of vascular BH4 levels may be an important role for the reversal of eNOS uncoupling. Taken together, AVE compounds exert overt vasoprotective effects through upregulating eNOS expression and reversing eNOS uncoupling.

3.4 Vascular endothelium impairment

3.4.1 Asymmetric dimethylarginine (ADMA) induced vascular endothelium impairment

Evidence has shown that the increased concentrations of circulating ADMA is associated with increased risk of cardiovascular disease, suggesting ADMA as an independent risk marker for all-cause mortality and cardiovascular disease^{56, 57}. Indeed, ADMA, a derivative of L-arginine, can competitively inhibit eNOS and thus diminish endothelium-derived synthesis of NO⁵⁸. Elevated ADMA levels cause a reduction of NO production and could consequently lead to endothelial dysfunction and cardiovascular events. In human studies, infusion of ADMA in healthy individuals led to reduced cardiac output and renal plasma flow, and to increased vascular resistance⁵⁹. Moreover, increased plasma ADMA levels were associated with increased oxidative stress^{60, 61}. In patients with coronary artery disease (CAD), serum

ADMA levels were positively correlated with eNOS uncoupling found in endothelium⁶².

Xue et al.²² investigated in porcine coronary small arteries function *in vitro* that pre-incubation with ADMA (100 μmol/L, 1 h) diminished bradykinin-induced relaxation, and reduced the NO release in response to bradykinin, and such reductions were restored by co-incubation with AVE3085 (10 μmol/L, 1 h). Furthermore, co-incubation of NOS inhibitor, Nω-nitro-L-arginine (L-NNA; with selectivity for eNOS and nNOS over iNOS), markedly decreased ADMA-induced superoxide generation in porcine arteries, suggesting ADMA induced eNOS uncoupling. In addition, co-incubation of AVE3085 attenuated ADMA-induced ROS and reactive nitrogen species formation. Importantly, coronary small arteries pre-incubated with ADMA and AVE3085, L-NNA did not further inhibit superoxide generation, suggesting the reversal of eNOS uncoupling was mediated by AVE3085. Interestingly co-incubation with AVE3085 in ADMA-treated arteries did not alter eNOS protein levels. However, AVE3085 increased eNOS activity by reversing the downregulation of p-eNOS^{Ser1177} and the upregulation of p-eNOS^{Thr495} induced by ADMA.

In contrast, Xuan et al.⁶³ demonstrated in left internal thoracic artery segments from patients undergoing coronary artery bypass grafting that 1-hr exposure to ADMA (100 μmol/L) resulted in impaired ACh-induced endothelium-dependent relaxation, decreased eNOS protein expression and increased superoxide production. Co-incubation with AVE3085 (30 μmol/L) restored endothelium-dependent relaxation, enhanced eNOS expression and reduced superoxide formation caused by ADMA.

The discrepancies between these two studies regarding AVE3085-mediated increased vascular eNOS expression may be due the difference of pathological condition of arteries (non-pathological porcine arteries vs arteries from patients with coronary artery disease) and dosage (10 vs 30 μmol/L). Thus, further studies are warranted to draw firm conclusions on the role of eNOS enhancement in ADMA-induced endothelial dysfunction.

3.4.2 Homocysteine (Hcy) induced vascular endothelium impairment

Increased plasma homocysteine (Hcy) has emerged as an independent risk factor for cardiovascular diseases⁶⁴. Evidence indicated that Hcy-induced oxidative stress was dependent on NOS activity, which was mainly attributed to uncoupled eNOS⁶⁵. Studies demonstrated that Hcy induced eNOS uncoupling through increasing superoxide generation and diminishing intracellular BH4 bioavailability^{65,66}, subsequently resulting in endothelial dysfunction.

Yang et al.²⁰ demonstrated in porcine coronary arteries-exposed Hcy that co-treatment with AVE3085 (10 μ mol/L, 24h) resulted in improved impairment of EDR to bradykinin, restored NO release, decreased superoxide generation, upregulated eNOS mRNA and protein expression, increased phosphorylation of eNOS at Ser1177 (p-eNOS^{Ser1177}), downregulated iNOS protein expression and normalized arginase activity. This beneficial effect of AVE3085 was attenuated when co-incubated with either Akt inhibitor (1L6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-sn-glycerocarbonate) or phosphoinositide (PI) 3 kinase inhibitors (LY294002 or wortmannin). These inhibitors did not affect eNOS expression that was upregulated by AVE3085, suggesting that the PI3 kinase/Akt pathway was involved in the eNOS activation related endothelial protection of AVE3085. In addition, co-incubation either with iNOS inhibitor (1400 W) or arginase inhibitor (nor- NOHA) in arteries-exposed to Hcy partially improved relaxation and NO release as well as normalized arginase activity. This was associated with blunted superoxide generation, suggesting that iNOS and arginase contributed to Hcy-induced endothelial dysfunction and superoxide formation. The NOS inhibitor, L-NNA, also lowered the superoxide level elevated by Hcy, suggesting the contribution of eNOS uncoupling to Hcy-induced superoxide generation. Importantly, co-treatment with AVE3085 in arteries-exposed to Hcy also blunted superoxide generation and this could not be reduced any further by L-NNA, indicating that AVE3085 reversed Hcy-induced eNOS uncoupling. Taken together, AVE3085 ameliorated Hcy-induced endothelial dysfunction by reversal eNOS uncoupling, downregulation of iNOS and inhibition of arginase activity and thus inhibited superoxide generation. AVE3085 enhanced NO production by upregulation of eNOS expression and by activation of eNOS activity through PI3 kinase/Akt-dependent pathway.

3.5 Cardiac ischemic injury

3.5.1 Ischemia-reperfusion (I/R)

Myocardial infarct is caused by the occlusion of coronary arteries due to plaque rupture. If this occlusion is temporary, a period of cardiac ischemia is followed by reperfusion and associated damage. Changes in eNOS expression and function are causal in this process. Decreased eNOS-derived NO presented in experimental MI⁶⁷ and patients with ischemic cardiomyopathy^{68, 69}. Also, genetic overexpression of eNOS in mice attenuated myocardial infarction after myocardial I/R⁷⁰, whereas eNOS-deficient mice showed an increased infarct size in such a model⁷¹, all signifying the important role of eNOS dysfunction in MI and I/R. Perkins et al.⁷² revealed in experimental I/R that co-treatment with an eNOS activity enhancer, protein kinase C epsilon type (PKC ϵ), and with BH4 restored the post-reperfused cardiac function. In contrast, co-treatment with PKC ϵ and BH2 resulted in enhanced uncoupled eNOS

activity, cardiac contractile dysfunction and increased ROS generation. Indeed, BH2, which lacks the capacity to act as eNOS cofactor, can competitively replace eNOS-bound BH4 and subsequently promote eNOS uncoupling⁴³. Together, this suggests that presence of BH4 is a prerequisite for therapeutic target eNOS activity enhancer, and increasing eNOS activity when eNOS is uncoupled, will result in deleterious consequences.

Frantz et al.²⁵ demonstrated in a mice model of coronary I/R (30 min/24h) that 7d pretreatment with AVE 9488 (30 mg/kg/day as dietary supplement) significantly increased eNOS expression levels and enhanced vasodilator- stimulated phosphoprotein (VASP) phosphorylation at Serine¹⁵⁷, a measure of NO bioavailability and NO/cGMP signaling pathway⁷³. This was associated with significantly decreased I/R injury, displayed by a smaller myocardial infarct size compared with the placebo group. In addition, levels of malondialdehyde (MDA), a lipid peroxidation marker, were reduced in mice that received AVE9488 pretreatment. Interestingly, AVE9488 did not alter myocardial BH4 levels in this study. Taken together, AVE9488 protects against cardiac I/R injury through augmentation of myocardial eNOS expression and activity.

3.5.2 Myocardial infarction (MI)

MI is typified by myocardial cell death due to prolonged ischemia. Long-term effects of MI are cardiac inflammatory and remodeling responses, including LV dilation, myocyte hypertrophy and interstitial fibrosis⁷⁴. Uncoupling of eNOS caused by BH4 oxidation is the main source of superoxide generation in the ventricular remodeling process after MI in a rats³². eNOS- deficient mice exhibited left ventricular dysfunction and modeling after MI, suggesting that enhancing cardiac eNOS expression may be beneficial to ameliorate MI⁷⁵. This observation was confirmed in mice with transgenic eNOS overexpression by the improved LV systolic and diastolic function, and attenuation of LV, myocyte hypertrophy⁷⁶, and myocardial infarct size⁷⁰ after experimental MI.

Likewise, the eNOS enhancer compounds improved cardiac function. Fraccarollo et al.³⁰ demonstrated in a rat model of MI that chronic AVE9488 treatment via dietary supplementation (approx. 1.25/kg/d, starting 7 days post MI for 9 wks) led to increased eNOS expression, restored eNOS activity, diminished vascular superoxide production, collectively translating in improved global cardiac function, and reduced pathological hypertrophy and pulmonary edema. AVE9488-treated MI rats displayed decreased ANP and BNP expression, markers of the hypertrophic response to MI. The AVE9488-mediated increase in eNOS-derived NO production may contribute to the antihypertrophic effects. Indeed, NO was seen to inhibit cardiomyocyte hypertrophy, via cGMP-dependent PKG activation⁷⁷, and to play an important role in the antihypertrophic effect of bradykinin⁷⁸. Besides, AVE9488-treated

MI in rats led to downregulated myocardial endothelin-1 (ET-1), possibly due to the inhibitory effect of NO on ET-1 expression and production^{79, 80}. Interestingly, in both studies AVE did not alter myocardial BH4 levels and eNOS dimer-to-monomer ratio. Thus, eNOS activity and the enhancer compounds ameliorate cardiac function after MI by preventing the increased cardiac fetal genes expression, augmenting NO production and improving endothelial vasomotor dysfunction, independent of eNOS uncoupling.

3.6 Heart failure

3.6.1 Diastolic heart failure (DHF)

DHF refers to heart failure with normal left ventricular (LV) ejection fraction, which features slow LV relaxation and increased LV stiffness⁸¹. It has been known that NOS-derived NO is also a crucial mediator of cardiac diastolic function⁸². Mice with triple deletion of all NOS isoforms show impaired diastolic function and cardiac hypertrophy⁸³. Importantly, diastolic dysfunction was found in mild hypertensive deoxycorticosterone acetate (DOCA)–salt treated mice and this was associated with marked eNOS uncoupling, as witness by increased ROS generation, decreased NOS-derived NO and depleted cardiac BH4 levels⁸⁴.

Westermann et al.²⁶ have demonstrated that oral administration of AVE3085 in a Dahl salt-sensitive rat model of DHF (10 mg/kg/day for 8 weeks) led to restored diastolic dysfunction, to reduced cardiac hypertrophy and fibrosis and to decreased myocardial oxidative stress. AVE3085 displayed clear anti-fibrotic effects. This was attributable to 1) dampening of calcineurin activation, 2) impairing extracellular ERK/Akt-mediated collagen production, and 3) by blunting the production of pro-fibrotic mediators, including transforming growth factor beta (TGF- β), connective tissue growth factor (CTGF). Moreover, AVE3085-treated DHF rats show downregulated iNOS and NOX subunits p22phox and gp91phox mRNA expression, possibly explaining the observed decrease in cardiac ROS generation.

Although the authors have shown enhanced eNOS in this particular study, it is unclear if AVE3085 attenuated the development of hypertension through a vascular action or has any independent effect on systolic and/or diastolic function of the failing heart⁸⁵. Besides, it would be interesting if further experiments investigate whether AVE3085 is able to recouple the uncoupled eNOS by measuring eNOS-dependent NO generation, eNOS dimer stability and enzymatic activity, and BH4/dihydrobiopterin (BH2) levels.

3.6.2 Left ventricular hypertrophy

It is well documented that uncoupling of eNOS is a prominent source of reactive oxygen species (ROS) generated in myocardium in pressure overload-induced ventricular remodeling and cardiac dysfunction^{86, 87}. Chen et al.³¹ demonstrated in a mouse model of pressure overload-induced left ventricular hypertrophy that oral administration of AVE3085 (10 mg/kg/day for 4 wks) resulted in increased cardiac eNOS mRNA and protein expression, leading to improved cardiac function, protected against cardiac hypertrophy and fibrosis, blunted the increases ANP and b-MHC mRNA expression and this was associated with attenuated the increases Smad2/3 protein expression and activation. This study suggests that AVE3085 inhibited Smad signaling pathway and thus attenuated cardiac remodeling. Taken together, AVE acts protective in both forms of heart failure, but the precise mode of action needs to be delineated further.

3.7 Arterial thrombosis and platelet activation

It has been suggested that the increased risk of thrombo-embolic events in congestive heart failure (CHF) is attributed to a hypercoagulable state⁸⁸, associated with abnormal platelet activity⁸⁹, impaired endothelium-dependent vasodilation and diminished release of endothelium-derived NO⁹⁰. Elevated levels of P-selectin⁹¹ and, interestingly, uncoupling of platelet eNOS⁹² have been found in patients with congestive heart failure. In *in vitro* study, overexpression of eNOS in bovine aortic endothelial cells (BAEC) inhibited platelet aggregation and this effect was abolished when treated with L-NAME, suggesting platelet aggregation was mediated by eNOS-dependent NO⁹³. Indeed, eNOS-derived NO plays an important role in inhibiting platelet activation through sGC-cGMP–PKG dependent signaling pathway (reviewed in⁹⁴). The activation of PKG by cGMP caused VASP-phosphorylation at Ser²³⁹, the cGMP/PKG preferred site, and further phosphorylate VASP at Ser¹⁵⁷⁷³. In platelets, phosphorylation of VASP correlated with vasodilator-induced inhibition of platelet activation and aggregation, as well as with inhibition of fibrinogen receptor activation^{95, 96}.

Schafer et al.³⁴ demonstrated in a rat model of chronic MI-induced severe congestive heart failure that long-term oral administration of AVE9488 25 ppm/day improved and normalized aortic NO generation, as assessed by the additional constriction of isometrically pre-constricted aortic rings in response to L-NNA. In addition, AVE9488-treated severe CHF rats displayed increased platelet VASP-phosphorylation at Ser¹⁵⁷ and Ser²³⁹, indicating the improvement of the activity of platelet inhibitors/inhibitory pathways and NO/cGMP pathway^{73, 95, 97}. Treatment with AVE9488 in severe CHF rats resulted in decreased platelet surface expression of P-selectin and glycoprotein 53, markers of platelet degranulation. The effect of AVE9488

mediated VASP-phosphorylation at Ser¹⁵⁷ was abolished in eNOS^{-/-} mice, suggesting that AVE 9488 was specific for eNOS. Thus, AVE9488 normalized the impaired platelet VASP-phosphorylation and in this way prevents platelet activation, suggesting a therapeutic benefit in thrombo-embolic complications in patients.

4 General conclusions

The key role of NO in cardiovascular protection is well established. The variety of NO effects *in vivo* mirrors its complex downstream signaling cascades, as NO can signal by cGMP-dependent and -independent pathways^{98,99}. NO generated by eNOS regulates blood pressure, vascular tone, leucocyte adhesion, cell proliferation and platelet aggregation, all critical processes in the pathogenesis of cardiovascular diseases. Therefore, agents that increase the bioavailability of eNOS-derived NO are attractive candidate therapeutics for the treatment of cardiovascular diseases. AVE9488 and AVE3085 have been identified as two novel structurally related small-molecular-weight compounds. Both act protective in cardiovascular disease by effecting eNOS up-regulation and by reversing eNOS uncoupling. Recent studies revealed clear beneficial effects of AVE compounds (table 2) in atherogenesis, in cardiac diseases and in vascular function, including improved endothelium-dependent relaxation, enhanced vascular barrier function, increased circulating EPC, reduced ADMA levels, inhibited MDA (oxidative stress index and inflammation), and disrupted superoxide levels generated by NOX and iNOS. These promising preclinical results merit further study of the long-term therapeutic benefit of AVE9488 and AVE 3085 in human disease.

Table 1. Phenotypic comparison of increased eNOS transgene expression vs AVE9488 treatment in a mouse model of atherosclerosis

Parameters	apoE ^{-/-} /eNOS Tg mice ¹⁰⁰	AVE 9488 treatment of apoE ^{-/-} mice ¹⁵
Expression of eNOS	Upregulation (+238%, aortic eNOS protein levels, Western blotting)	(10 mg/kg/day b.i.d. for 17 days) Upregulation (+109%), aortic eNOS protein levels, Western blotting)
Functional status of eNOS	eNOS uncoupling	Recoupling of uncoupled eNOS
NO production	Increase (+50%)	n/a
BH4 levels	No effect (52% decrease compared with WT)	Increase (+80%)
Superoxide formation	Increase (+75% (non-atherosclerotic artery segment); + 30% in plaque	Decrease (-33%)
Atherosclerotic lesion formation	Increase (+ 50%) 12 wk of high cholesterol diet (1.25% cholesterol, 7.5% cocoa butter, 7.5% casein, 0.5% sodium cholate)	Decrease (30 mg/kg/day for 12 wk) <ul style="list-style-type: none"> • Chow: plaque formation (-39%); plaque area (-32%) • Western-type diet (20% fat and 0.5% cholesterol): plaque formation (-36%) and for AVE3085 treatment :-45%

Table 2. Overview of major vascular and myocardial preclinical studies with AVE compounds.*

Cardiovascular dysfunction	Model	Dosage of AVE compounds	Major findings	Ref.
Hypertension and hypertension-induced endothelial dysfunction.	SHR rats (in vivo)	AVE3085 (10mg/kg/day; 4wks; oral gavage)	<ul style="list-style-type: none"> - Improved endothelium-dependent relaxation (EDR) in aortas - Up-regulated eNOS mRNA and protein - Enhanced eNOS phosphorylation - Decreased nitrotyrosine formation - Reduced blood pressure 	29
	SHR rats (ex vivo- aorta)	AVE3085 10 μ M; 2hr incubation	<ul style="list-style-type: none"> - Improved EDR in aortas - Up-regulated eNOS mRNA - Enhanced eNOS phosphorylation 	
	SHR rats (in vitro; primary endothelial cells from aortas)	AVE3085 10 μ M; 12 hr incubation	<ul style="list-style-type: none"> - Up-regulated eNOS mRNA 	
Vasoreperative dysfunction in diabetic EPC	CD34+ isolated from peripheral blood from diabetic patients	AVE3085 (10 μ M; 24 hr incubation)	<ul style="list-style-type: none"> - Increased eNOS mRNA expression resulted in increased peroxynitrite levels and, therefore, did not enhance NO-mediated function <i>in vitro</i> and <i>in vivo</i> - Migration in response to SDF-1α remained impaired - No improvements of vasoreperative function in diabetic CD34+cells. 	24
	A mouse model of retinal I/R	AVE3085 (10 μ M; 24hr) AVE-treated diabetic CD34+cells	<ul style="list-style-type: none"> - AVE3085-treated diabetic cells did not migrate to areas of vascular injury when injected within the vitreous of mice undergoing I/R of retinal injury. 	

Cardiovascular dysfunction	Model	Dosage of AVE compounds	Major findings	Ref.
Endothelial dysfunction in diabetes	Diabetic db/db mice	AVE3085 (10 mg/kg/day; 7days; p.o.)	<ul style="list-style-type: none"> - Increased eNOS level but did not change the level of p-eNOS^{Ser1176} to total eNOS. The ratio of p-eNOS^{Thr495} to eNOS was not changed in db/db mice when compared with control or by AVE3085 treatment - Reduced blood pressure - Improved EDR to ACh in aortas, mesenteric and renal arteries - Lowered ROS generation - Augmented the attenuated flow- dependent dilatation in mesenteric resistance arteries 	23
	High-glucose–incubated mouse aortas (HG 30mmol/L; 48hrs)	AVE3085 (1μM; incubation)	<ul style="list-style-type: none"> - Increased eNOS expression - Improved EDR to ACh in aortas, mesenteric and renal arteries - Inhibited ROS generation 	
Atherosclerosis	EA.hy 926 cells	AVE9488 and AVE3085 10 μM; 18 h; incubation	<ul style="list-style-type: none"> - Stimulated eNOS promoter activity in a concentration-dependent manner without changing eNOS mRNA stability 	15
	primary human umbilical vein endothelial cells	AVE9488 2 μM; 18 h; incubation	<ul style="list-style-type: none"> - Increased eNOS mRNA and protein expression - Enhanced bradykinin-stimulated release of NO 	
	C57BL/6J apoE ^{-/-} mice	AVE9488 (30 mg/kg/day; 17 days; pressed in chow)	<ul style="list-style-type: none"> - Enhanced vascular eNOS expression (femoral arteries) 	
	apoE ^{-/-} mice with cuff-induced neointima formation	AVE9488 (10 mg/kg/day; b.i.d. starting 3 days before the cuff placement 17 days;)	<ul style="list-style-type: none"> - Reduced neointima formation - Enhanced aortic eNOS expression 	

Cardiovascular dysfunction	Model	Dosage of AVE compounds	Major findings	Ref.
Atherosclerosis (continued)	apoE ^{-/-} mice (standard rodent chow)	AVE9488 10 (30mg/kg/day; 12 wks)	<ul style="list-style-type: none"> - Reduced aortic plaque formation - Enhanced eNOS protein expression (femoral arteries) 	
	apoE ^{-/-} mice (western type diet)	AVE9488 or AVE3085 (30mg/kg/day; each; 12 wks)	<ul style="list-style-type: none"> - Reduced aortic plaque formation 	
	apoE ^{-/-} mice	AVE9488 (30mg/kg/day; 2wks)	<ul style="list-style-type: none"> - Increase aortic BH4 levels - Decreased aortic ROS production - Reversed eNOS uncoupling 	
Asymmetric dimethylarginine (ADMA) induced vascular endothelium impairment.	Left internal thoracic artery segments from patients undergoing coronary artery bypass grafting incubated with ADMA 100 µmol/L	AVE3085 (30 µM; incubation)	<ul style="list-style-type: none"> - Improved EDR - Increased vascular eNOS expression - Decreased ADMA- induced ROS production 	22
Homocysteine (Hcy) induced vascular endothelium impairment	Porcine Coronary Small Arteries (PCAs) incubated with Hcy 50 µmol/L	AVE3085 (10 µM; 24h; incubation)	<ul style="list-style-type: none"> - Improved EDR to bradykinin - Increased bradykinin-stimulated NO release - Decreased ROS generation - Increased eNOS expression both mRNA and protein levels and p-eNOS^{Ser1177}, increased p-eNOS^{Ser1177}:eNOS ratio - Blunted the increased iNOS protein expression - Normalized the arginase activity 	20
Cardiac ischemia injury	Ischemia reperfusion: mice model of coronary I/R	AVE9488 (30 mg/kg/day; 7days; dietary supplement)	<ul style="list-style-type: none"> - Increased eNOS expression - Increased VASP phosphorylation - Decreased myocardial infarctions (infarct/ area at risk) - Decreased malondialdehyde-thiobarbituric acid (MDA) 	25

Cardiovascular dysfunction	Model	Dosage of AVE compounds	Major findings	Ref.
Cardiac ischemia injury (continued)	Chronic myocardial infarction: rat model of MI	AVE9488 (25 ppm AVE 9488 as a dietary supplement ; average dose is 1.25/kg/d, starting 7 days after coronary artery ligation for a period of 9 wks)	<ul style="list-style-type: none"> - Increased aortic and myocardial eNOS protein levels - Increased calcium-dependent NOS activity in left ventricular myocardium - Improved EDR in aortas - Prevented LV dilatation, hypertrophy, fibrosis - Blunted the increased in cardiac gene expression (ANP, BNP and ET-1) - Decrease ROS generation - Reduced myocardial nitrotyrosine levels - Increase p-VASP at Ser 239 - Increased circulating EPC 	30
	Hypoxia reoxygenation: porcine coronary small arteries incubated with St. Thomas solution	AVE3085 (10 μ M; incubation)	<ul style="list-style-type: none"> - Preserved the bradykinin induced EDR - Enhanced eNOS expression - Increased phosphorylated eNOS at Ser 1177 (p-eNOS Ser1177) 	21
Impairment of bone marrow mononuclear cells (BMC) in ischemic cardiomyopathy (ICMP) and cell therapy	BMC derived from patients with ICMP (ex vivo and in vitro)	AVE9488 (5 μ M 18-24hr; incubation)	<ul style="list-style-type: none"> - Increased eNOS mRNA expression in peripheral blood-derived EPC, CD34+ and BMC - Increased eNOS protein expression and activity, and NO production in Lin-CD105+ - Increased migratory capacity of BMC in response to stromal cell-derived factor 1 (SDF-1) 	14
	Murine BMC	AVE9488 (5 μ M 18 hr; incubation)	<ul style="list-style-type: none"> - Increased migratory capacity of BMC in response to SDF-1 in WT 	
	A nude mouse model of unilateral hind limb ischemia (in vivo)	AVE9488 (5 μ M 18 hr; incubation; AVE-treated BMC from patients with ICMP; i.v. infusion)	<ul style="list-style-type: none"> - Improved neovascularization capacity - Enhanced recovery of limb perfusion - Increased the swimming time ratio 	

Cardiovascular dysfunction	Model	Dosage of AVE compounds	Major findings	Ref.
Heart failure	DAHL salt-sensitive induced Diastolic heart failure (DHF) rats	AVE3085 (10mg/kg/day; 8wks; p.o.)	<ul style="list-style-type: none"> - Increased eNOS mRNA and protein - Decreased NADPH oxidase (NOX) subunits p22phox and gp91phox - Decreased iNOS gene expression - Decreased cardiac ADMA levels - Attenuated diastolic dysfunction - Reduced cardiac hypertrophy and fibrosis - Normalized activation state of calcineurin, AKT and ERK 1/2 	26
	Pressure overload-induced left ventricular hypertrophy mice	AVE3085 (10 mg/ kg /day, 4 wks; p.o.)	<ul style="list-style-type: none"> - Increased eNOS expression - improved cardiac function - Alleviated cardiac remodeling - Downregulated ANP and b-MHC mRNA expression - Inhibited Smad1/2 signaling pathway 	31
Arterial thrombosis and platelet activation	congestive heart failure rats (CHF; left ventricular end-diastolic pressure >15 mmHg)	AVE9488 (25 ppm/day); 10 wks; p.o. as diet supplements)	<ul style="list-style-type: none"> - normalized platelet VASP phosphorylation at serine 157 and 293 in eNOS-dependent manner - reduced expression of markers of platelet degranulation (i.e. P-selection and glycoprotein 53) 	34

(*) Of note, the beneficial effects in enhancing eNOS expression and activity of AVE compounds are absent in the presence of NOS inhibitor substances (L-NAME and L-NNA), transcription inhibitor (actinomycin D), endothelium-denuded rat aorta, and in eNOS^{-/-} mice)

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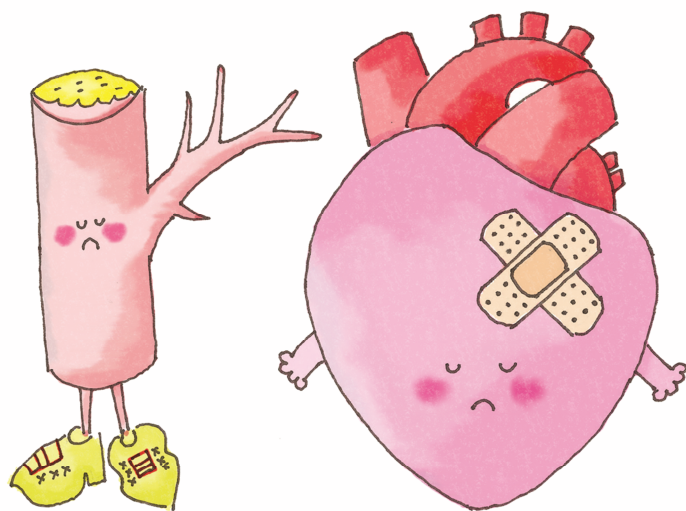
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Chapter 5

(Pharmacological) Enhancement of endothelial nitric oxide synthase activity prevents pressure overload-induced ventricular hypertrophy

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(In preparation)

Abstract

Aims: Endothelial nitric oxide synthase (eNOS) has a central role in the pathogenesis of pressure overload-induced ventricular remodeling. The small molecule AVE3085 can enhance transcription of eNOS and interference with eNOS-uncoupling has been suggested. In this study, we tested if AVE3085 can prevent pressure overload-induced cardiac hypertrophy.

Methods and results: Mice C57BL/6 (n=12/group) underwent severe transverse aortic constriction (TAC) for one week to develop cardiac hypertrophy and remodeling. Oral AVE3085 (30mg/kg/day) or placebo (0.5% methyl hydroxyethylcellulose) were given from the day of surgery for 7 days. AVE3085 treatment reversed TAC-induced cardiac hypertrophy indices, including heart weight/body weight ratio (HW/BW; -21%), heart weight/ tibia length ratio (HW/TL; -13%), myocyte dimension (-9%) and relative fibrosis area (-60%). Also, hypertrophic markers B-type natriuretic peptide (BNP) and beta myosin heavy chain (bMHC) expression were normalized by - 6% and -3% respectively compared to control TAC mice. This was associated with improved cardiac function, including left ventricular end-systolic diameter (LVESD; -34%), wall thickness (-24%), calculated LV mass (-76%), fraction shortening (FS; 15%), and ejection fraction (EF; 7%). Moreover, a reduction in cardiac reactive oxygen species (ROS) compared to control TAC mice was not reduced further by the NOS inhibitor L-NAME. Interestingly, eNOS homodimer stability was also improved. Together this suggests AVE3084 reversed eNOS uncoupling, while eNOS mRNA and protein levels only showed a trend to increase after AVE3084 treatment.

Conclusion: AVE308 treatment reversed pressure overload-induced ventricular remodeling, which was likely mediated by a reversal of eNOS uncoupling through protection of tetrahydrobiopterin oxidation by ROS.

Introduction

Sustained pressure overload can lead to pathologic cardiac hypertrophy and dysfunction¹, and prevention or delay of this maladaptative response has emerged as an important therapeutic goal². Under physiological conditions, nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS) plays a key role in cardiomyocyte contractility and vascular tone^{3,4}. Proper function of eNOS requires dimerization of the enzyme and the presence of its cofactor tetrahydrobiopterin (BH4). Functional eNOS is able to oxidize its substrate L-arginine to produce L-citrulline and NO. It is well documented that uncoupling of eNOS is a prominent source of reactive oxygen species (ROS) generated in myocardium in pressure overload-induced ventricular remodeling and cardiac dysfunction^{5,6}. eNOS uncoupling involves a shift from dimeric to monomeric state, resulting in reduced NO production and increased ROS generation⁷. The production of ROS in turn enhances oxidation of BH4, leading to decrease bioavailability of this cofactor, creating a vicious cycle of progressive eNOS uncoupling. Although BH4 deficiency is a major cause for eNOS function loss⁸, diminished eNOS transcription, mRNA stability and translation were proposed to be instrumental in this process (see reviews⁹). Because uncoupled eNOS generates not only NO, but also ROS, increasing eNOS transcription, translation or activity does not guarantee improved endothelial function¹⁰. Hence pharmacological approaches to preserve or restore eNOS functionality and prevent eNOS uncoupling are thus warranted. Indan compounds AVE9488 and AVE3085 were designed to enhance eNOS gene expression, with the eventual aim to increase NO signaling and bioavailability^{11,12}. Surprisingly, AVE 9488 has also been shown to enhance endothelial BH4 levels and reverse eNOS uncoupling in experimental atherosclerosis¹². This favorable activity profile suggests that AVE compounds could be effective in eNOS dysfunction induced cardiac diseases. While AVE3085 was seen to prevent cardiac remodeling in experimental compensated left ventricular dilation¹³, its effect on eNOS dysfunction and myocardial ROS formation in a mouse model of left ventricular hypertrophy is unknown. To investigate the potential role of AVE3085, a novel pharmacological modulator of eNOS, in cardiac stress remodeling associated with pressure overload, we subjected mice to severe transverse aortic constriction (TAC) for 1 week as a model of acute onset of HF. It is well established that eNOS uncoupling plays a major role in TAC pathobiology. Moreover, mice undergoing severe, but not mild, TAC show enhanced eNOS uncoupling associated with increased eNOS-dependent oxidative stress, and consequent cardiac dysfunction and remodeling^{5,6}. We used C57BL/6 mice because they develop rapid cardiac hypertrophy, myocyte hypertrophy and fibrosis within 7 days after TAC^{7,8}, allowing us to examine the utility of pharmacological or molecular interventions that may limit hypertrophy. In this study we therefore investigated the effects of AVE3085 on

pressure overload induced eNOS uncoupling, and subsequent prevention and/or reversion of left ventricular hypertrophy.

Materials and Methods

All experiments were approved by the Animal Experiment Committee of Maastricht University and were performed in accordance with institutional and international guidelines.

TAC and Study Protocols

36 Male C57BL/6 mice (n= 12 per group; age, 9 to 11 weeks; weight, 22 to 24 g; Charles River, Maastricht, the Netherlands) underwent sham or severe transverse aortic constriction (TAC) surgery as previously described⁵. The mice were randomized to receive AVE3085 (30mg/kg/d; in 0.5% methyl hydroxyethylcellulose (vehicle)) or placebo as a 7-day p.o. treatment, starting at the day of surgery. Animals were sacrificed at day 7 and organs were harvested for molecular, cellular, and superoxide anion generation. The experimental setup of this study is depicted in figure 1A.

Cardiac Function

In vivo cardiac morphology and function was assessed by transthoracic echocardiography; using a Hewlett-Packard 15 MHz linear array transducer (15-6L) interfaced with a Sonos 5500 echocardiography system (Phillips, Eindhoven, The Netherlands) in conscious mice at baseline and at the end of the experiment. M-mode left ventricular (LV) end-systolic and end-diastolic dimensions were averaged from 3 to 5 beats, and data were analyzed by investigators blinded to the heart condition as described⁵.

Histology

The myocardium was fixed in 10% formalin, dehydrated in alcohol, embedded in paraffin, and 5 µm sections were made. Subsequently, hematoxylin and eosin (HE) staining was performed. Images were taken with a Leica microscope at 20x magnification using QwinV3 software. Myocyte cross-sectional diameter was measured at the level of the nucleus in the longitudinally sectioned slides and analyzed with Adobe Photoshop 7.0.1 (3 slices in 4 different hearts in each group, averaging results from 60-90 cells per group). Interstitial fibrosis was measured using picro-sirius red (0.05% in saturated picric acid solution, Sigma). Images of non-vascular areas were randomly captured (4-5 fields per heart; n=4 per group; 20x magnification). Relative fibrosis areas were quantified by ImageJ MRI fibrosis tool (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Fibrosis_Tool).

Gene expression analysis

Total mRNA was extracted from 4-5 frozen LV samples per group using the RNeasy kit (Qiagen, The Netherlands) according to the manufacturer's protocol. RT-qPCR was performed for specific genes of using SensiMix SYBR Green (Bio-Rad) on a Bio-Rad CFX96 Real-Time System, C1000 Thermal Cycler. Primer sequences for myocardial hypertrophy marker genes A- and B-type natriuretic peptide (ANP and BNP), alpha and beta myosin heavy chain (αMHC and βMHC), and phospholamban (PLB) are shown in table 1. mRNA expression were expressed relative to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized to placebo-treated expression values.

mRNA expression of eNOS and guanosine triphosphate cyclohydrolase I (GCH1) was analyzed with the QuantiTect Probe RT-PCR kit (QIAGEN GmbH). TaqMan gene expression assays (predesigned probe and primer sets) were obtained from Applied Biosystems (Foster City, CA): eNOS (nos3; assay ID Mm00435204_m1), GCH1 (assay ID Mm00514993_m1) and GAPDH (assay ID 4352339E). Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

eNOS protein expression and dimer to monomer ratio

Protein expression of eNOS was determined in homogenates of myocardium samples (n=5-6 per group) as previously described ¹⁴. Low temperature sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for detection of eNOS monomer and dimer as previous described ⁶. Cold SDS-PAGE Western blot analysis was performed in 7% to 4% SDS-Tris gels run overnight on ice and then transferred for 3 hours to nitrocellulose membranes. Primary eNOS antibody (1:1000, Santa Cruz Technology, Inc, Santa Cruz, Calif) was detected by enhanced chemiluminescence (Pierce, Rockford, Ill) using a LAS 3000 imaging system. All protein bands were quantified using ImageJ (NIH).

Superoxide detection

Superoxide generation in homogenized LV (n= 3 per group) was evaluated by low concentration lucigenin-enhanced chemiluminescence (5 μmol/L) using a single-tube luminometer (Berthold FB12) as previously described ¹⁵. Besides total superoxide production, NOS-dependent ROS production was determined by incubation of the same homogenized LV samples for 30 min with the NOS inhibitor L-NAME (Sigma-Aldrich) (1 mmol/l). During the entire experiment the temperature was maintained at 37 °C; light emission was recorded and expressed as relative light units (RLU) per mg per second ¹⁶.

2.8 Statistical Analysis

All data are expressed as mean \pm SEM. Following the Kolmogorov-Smirnov test for normal distribution, the two groups were compared with student's t-test (Gaussian distribution) or Mann-Whitney test (non-Gaussian distribution). Parameters with more than two groups were analysed using one way ANOVA in combination with a Bonferroni post-hoc analysis, or Kruskal-Wallis Test, followed by Dunn's post-hoc testing. Analyses were performed using GraphPad Prism 5.0 (La Jolla, CA, USA). $P < 0.05$ was considered to be statistically significant.

Results

AVE3085 reversed cardiac hypertrophic remodeling and fibrosis

To assess the effect of AVE3085 on left ventricular hypertrophy, histological analysis of the heart tissue was performed and cardiac myocyte dimension and interstitial fibrosis were measured (Figure 1B-I). As expected, one week after TAC surgery in the placebo treated group we observed overt signs of cardiac hypertrophy, including heart weight /body weight ratio (HW/BW; 70%), heart weight/ tibia length ratio (HW/TL; 49%), myocyte dimension (37%) and relative fibrosis area (1870%), compared to the sham-operated group. As shown in Figure 1B-I, hypertrophy reversed and heart function improved in TAC mice that had received AVE3085, as evidenced by a decreased hypertrophic response, including HW/BW (-21%), HW/TL (-13%), myocyte dimension (-9%) and relative fibrosis area (-60%). Treatment with AVE3085 decreased the TAC associated induction of the expression of hypertrophic markers B-type natriuretic peptide (BNP) and beta myosin heavy chain (bMHC) (Figure 1I). Together, these findings indicated that AVE3085 was able to reversed cardiac hypertrophy and remodeling after pressure overload.

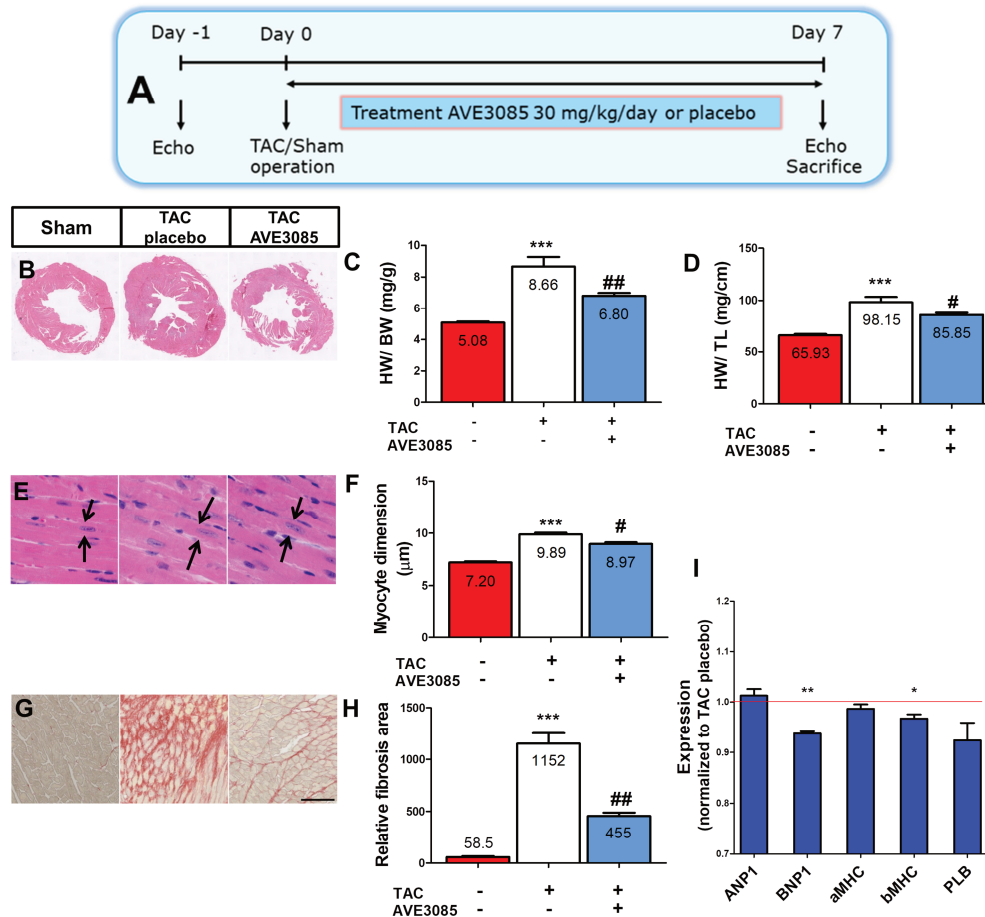


Figure 1. AVE3085 reverses cardiac hypertrophic remodeling and fibrosis

A. Schematic representation of experimental procedure for pressure overload-induced acute onset of heart failure in mice. **B.** Representative haematoxylin and eosin (HE) stained cross-sections of the murine heart 1 week after sham, TAC operation and/or AVE treatment (magnification = 1x), and corresponding quantifications of **C.** Mean data for heart weight to body weight ratio (HW/BW), and **D.** Heart weight to tibia length ratio (HW/TL). **E.** Representative HE staining showing myocyte dimensions (black arrows indicate dimensions; Magnification = 20x) and **F.** corresponding quantifications (n = 4 group; 3 regions per heart averaging results from 60-90 cells per group). **G.** Picro-sirius red (PSR) staining revealed increased interstitial fibrosis in placebo-treated TAC heart (red staining; n=4 per group; 4-5 fields per heart; 20x magnification scale bar 50 μm). **H.** Quantification of relative fibrosis area, showing reversed interstitial fibrosis in AVE3085-treated TAC mice. ***p<0.001 vs. sham placebo; #p<0.05, ##p<0.01 vs. TAC placebo. **I.** Analysis of fetal gene mRNA expression in TAC hearts, normalized by TAC placebo group (n=5 per group). *p<0.05, **p<0.01 vs TAC placebo. A- and B-type natriuretic peptide (ANP and BNP), alpha and beta myosin heavy chain (αMHC and βMHC), and phospholamban (PLB).

AVE3085 improved cardiac function

Cardiac function was assessed by M-mode echocardiography in conscious mice. As shown in (Figure 2A-H), one week TAC resulted in increased left ventricular end-systolic diameter (LVESD; 110%), end-diastolic wall thickness (90%) and calculated left ventricular (LV) mass (208%), while reducing cardiac contractility as assessed by fraction shortening (FS; -19%) and ejection fraction (EF; -9%), compared to sham operated placebo treatment. Treatment with AVE3085 attenuated all these deleterious changes (Figure 2A-H), with marked decreases in LVESD (-34%), wall thickness (-24%) and calculated LV mass (-76%) compared to placebo-treated TAC mice. A minor but significant increase in cardiac contractility parameters was detected (FS: 15% and EF: 7%), compared to placebo-treated TAC mice. Thus, AVE3085 diminished pathological LV remodeling and myocardial contractile dysfunction caused by pressure overload- induced LV hypertrophy.

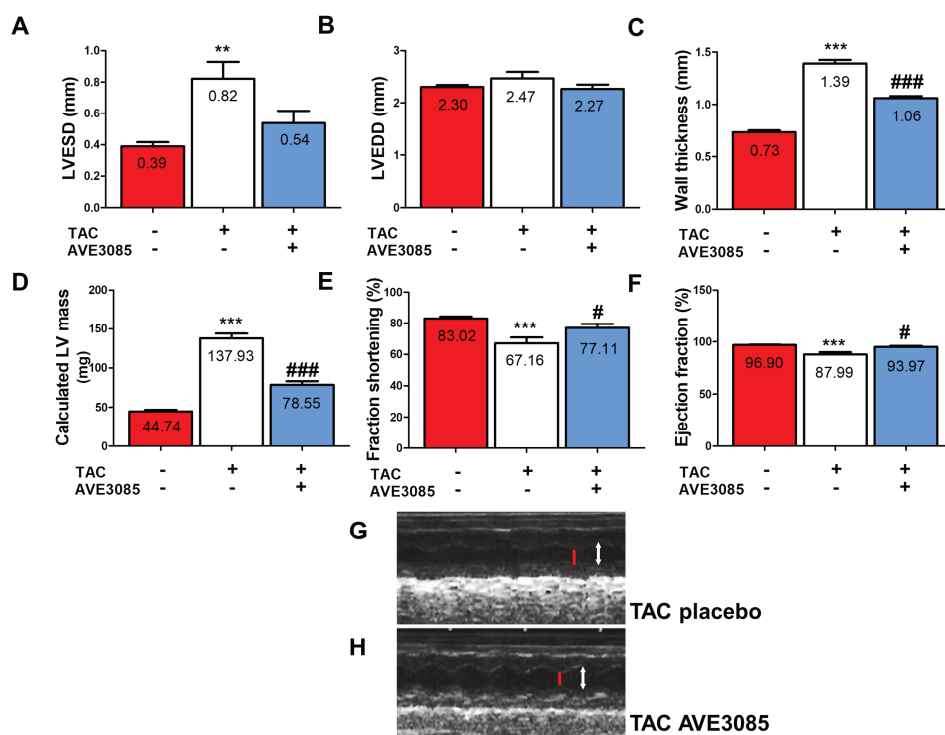


Figure 2. AVE3085 improves cardiac function

*AVE3085 improves cardiac function assessed by M-mode echocardiography in conscious mice, as shown by A. left ventricular end-systolic diameter (LVESD; mm), B. left ventricular end-diastolic diameter (LVEDD; mm), C. end-diastolic wall thickness (mm), D. calculated left ventricular (LV) mass (mg); E. fraction shortening (FS; %), and F. ejection fraction (EF; %). ** $p < 0.01$, *** $p < 0.001$ vs. sham placebo; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. TAC placebo. G,H. Representative example of M-mode echocardiography in conscious animals, showing LVESD (red line) and LVEDD (white arrow)*

AVE3085 enhanced functional eNOS dimer formation and reversed myocardial eNOS uncoupling.

To dissect AVE's mode of action, we determined eNOS mRNA expression levels (RT-qPCR) and protein expression (Western blotting) in homogenized heart. As shown in figure 3A-B, treatment with AVE3085 in TAC mice tended to increase eNOS mRNA and protein expression compared to placebo-treated TAC mice, albeit it that these effects were not significant. It has been suggested that eNOS homodimer instability, reflected by a lower ratio of dimers to monomers, may indicate eNOS uncoupling^{5, 6}. Surprisingly, although treatment with AVE3085 did not change total eNOS protein levels, it led to elevated eNOS dimer-to-monomer ratio (Figure 3C). This suggests that AVE3085 has promoted eNOS dimerization and thus enhanced functional eNOS expression in TAC mice. In contrast, placebo-treated TAC group exhibited a lower dimer-to-monomer ratio, suggestive of eNOS uncoupling and elevation of the ROS producing eNOS monomer.

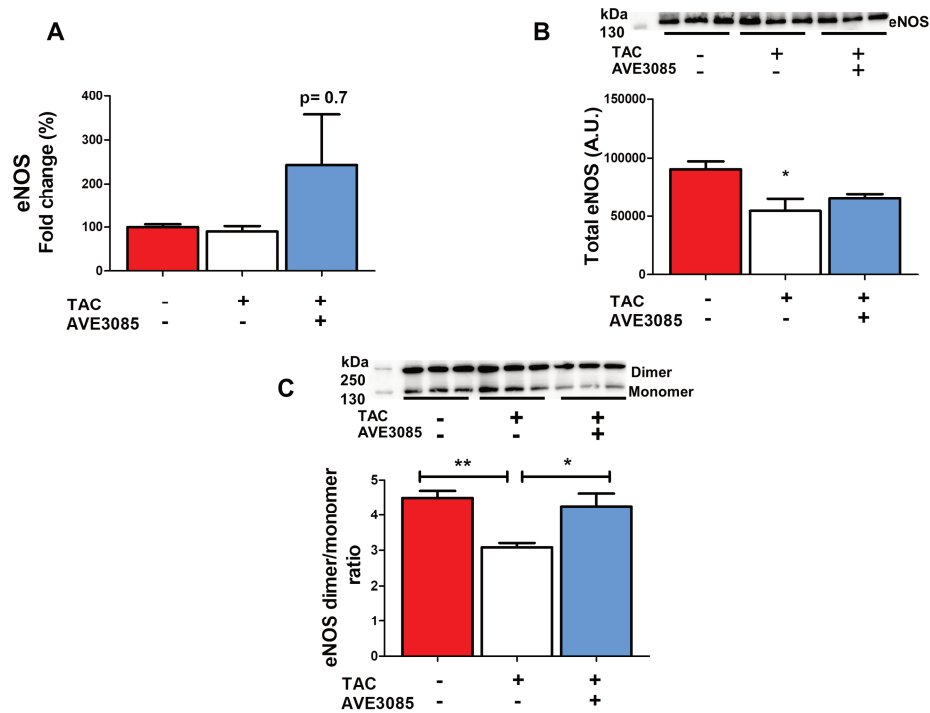


Figure 3. AVE3085 enhances functional eNOS dimer formation

A. The eNOS mRNA level 1 week after TAC was normalized to the reference gene (GAPDH) using the $2^{-\Delta\Delta CT}$ method ($n=5-6$ per group). **B.** Representative western blot and quantification of total eNOS protein expression ($n=5-6$ per group). **C.** Representative western blot and quantification of eNOS dimer and monomer protein expression (dimer to monomer ratio; $n=5$ per group).

Indeed, AVE3085 enhanced myocardial superoxide generation, as assessed by lucigenin-enhanced chemiluminescent in LV homogenates. As shown in figure 4A, one week TAC substantially stimulated basal myocardial ROS formation in the placebo-treated group. Co-incubation of LV homogenates with the NOS inhibitor, L-NAME resulted in a significant decrease in ROS generation, suggesting that cardiac ROS were at least partially attributable to eNOS uncoupling. These findings were supported by our observation in placebo-treated TAC mice, which displayed a decrease dimer-to-monomer ratio and thus monomeric eNOS contributed to increases ROS production. In contrast, treatment TAC mice with AVE3085 led to lowered myocardial ROS production. Under these conditions, L-NAME did not further reduce myocardial ROS production in AVE3085-treated group, indicating that AVE3085 reversed eNOS uncoupling.

We further studied if eNOS uncoupling was dependent on BH₄⁶. The rate-limiting step of BH₄ biosynthesis is initial guanosine triphosphate (GTP) modification, catalyzed by the enzyme GTP cyclohydrolase I (GCH1)¹². Indeed, treatment with AVE3085 tended to induce GCH1 mRNA expression in TAC mice (Figure 4B) vs placebo-treated TAC group, suggestive of enhanced BH₄ biosynthesis. Taken together, these findings suggested the AVE3085 maintained eNOS homodimer stability, reduced myocardial ROS production and subsequently prevented or reversed eNOS uncoupling induced by pressure overload.

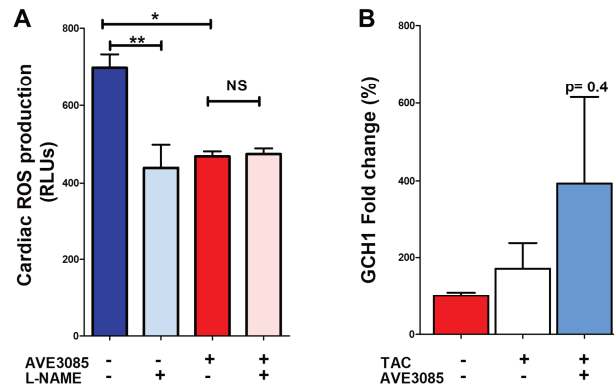


Figure 4. AVE3085 reverses myocardial eNOS uncoupling

A. Cardiac superoxide anion production was measured by lucigenin-enhanced chemiluminescence and expressed as the relative light unit (RLUs). Experiments were performed in the absence or presence of the NOS inhibitor 1 mmol/l L-NAME ($n=3$ per group). * $p<0.05$, ** $p<0.01$, NS not significant. **B.** The GCH1 mRNA level 1 week after TAC was normalized to the reference gene (GAPDH) using the $2^{-\Delta\Delta CT}$ method ($n=5-6$ per group).

Discussion

In the present study, we are the first to investigate the role of AVE3085 in modulating eNOS functionality in pressure overload-induced acute onset of LV hypertrophy in mice. Our main findings suggested clear beneficial effects of AVE3085 treatment as it (1) reverses cardiac hypertrophy, interstitial fibrosis and blunted re-expression of fetal genes, 2) attenuates the pathological LV remodeling and myocardial contractile dysfunction, assessed by M-mode echocardiography, and 3) mechanistically, preserves eNOS homodimer stability even in the absence of transcriptional enhanced eNOS, and reduces myocardial ROS production. Taken together this suggests AVE3085 prevents and/or reverses eNOS uncoupling induced by pressure overload.

The observation that, AVE 3085 attenuated the pathological LV changes associated pressure overload, in the absence of transcriptionally enhanced eNOS protein and mRNA expression is surprising as AVE3085 treatment (10mg/kg/day for 7days) was previously shown to increase aortic eNOS expression in diabetic mice ¹⁷. At the same dose, AVE3085 led to increased cardiac eNOS mRNA and protein expression in mice subjected to pressure overload for 4 week ¹³. A potential explanation for AVE3085's failure to enhance eNOS expression is a compensatory increase in eNOS levels in the early stages of hypertension due to decreased NO production, resulting from increased ROS production ¹⁸. Therefore, eNOS protein levels may be similar between groups. Alternatively, AVE3085 may not have penetrated the intracellular compartments of cardiac tissue. Therefore, pharmacokinetics analysis and intracellular AVE3085 handling in myocardium in TAC mice is warranted. If myocardial bioavailability of AVE3085 is compromised, AVE3085 may exert its pleiotropic effects ectopically by lowering blood pressure, improving arterial endothelium-dependent relaxation ¹⁹, and/or by downregulation oxidant enzymes ²⁰ as it has demonstrated in spontaneously hypertensive rats and experimental diastolic heart failure, respectively. AVE3085 mediated peripheral vascular function improvement may thus have led to an improvement of cardiac function in TAC mice.

Interestingly, instead of transcriptional upregulation, our study has unveiled a beneficial effect of AVE3085 treatment on eNOS functional stability, demonstrated by a concomitant increase in eNOS dimer-to-monomer ratio and by decreased myocardial ROS generation. The observation that AVE3080 lowered TAC-induced ROS generation independent of NOS inhibitor L-NAME further supports such reversal of eNOS uncoupling. Our findings were in line with previous studies those demonstrated that AVE9488 and AVE3085 can reverse eNOS uncoupling ^{12, 21, 22}. It is well documented that uncoupling of eNOS, but not iNOS, is a prominent source of

myocardial ROS and contributes in this way to cardiac dysfunction and remodeling⁵. Indeed in severe and sustained pressure overload (≥ 3 weeks), lack of eNOS ameliorated^{5, 16}, whereas eNOS overexpression exacerbated, TAC-induced cardiac remodeling and dysfunction. The latter was associated with increased myocardial ROS production and enhanced eNOS uncoupling¹⁶. In line with our studies, this indicates that while functional eNOS generally acts protective against maladaptive stress responses, once uncoupled, it becomes a major ROS generator, contributing to the pathophysiology.

Since BH4 is highly susceptible to oxidative degradation caused by ROS⁸ and AVE3085 treatment attenuated myocardial ROS generation in TAC mice, suggesting the beneficial effect of AVE3085 is likely the result of protection of BH4 oxidation by ROS. It is indeed known that BH4 increases the stability of the eNOS homodimer, and the stoichiometry of BH4–eNOS interaction is directly related to eNOS coupling^{23, 24}. Exogenous administration of BH4 has been shown to reverse pre-established advanced hypertrophy and decompensated dilation, which aligns with our AVE3085 findings for cardiac hypertrophy (1 week treatment).

In contrast, BH4 administration from TAC onset or cardiomyocyte specific guanosine triphosphate cyclohydrolase I (GCH) -overexpression did not restore cardiac function, assessed by echocardiogram analysis after 1 week of TAC^{6, 25}. The former may be due to eNOS coupling by overexpressed or supplemental BH4²⁵. Previous studies have demonstrated that 1 week of TAC did not alter endogenous cardiac BH4 levels²⁶ or affect eNOS activity⁶. This suggests that eNOS is functional and can still generate NO production in the presence of sufficient BH4^{8, 27}. Together, this might explain why BH4 treatments do not show beneficial effect after 1 week.

Although the capacity of AVE3085 to reverse pre-existing disease remains to be investigated, AVE3085 appears to protect BH4 from oxidation by ROS, when administered at the onset of pressure overload-induced cardiac hypertrophy. The mechanisms for the increase dimeric eNOS and BH4 mediated eNOS homodimer stability still remain to be addressed.

We are aware of the limitations of our study. First, we have not included a sham-operated group that received AVE3085, as previous studies had already demonstrated that AVE3085 treatment of control mice without surgery did not impact relevant hemodynamics, blood pressure and body weight^{20, 28}. Second, cardiac BH4 and BH2 levels remain to be assessed to fully underpin that BH4 dependent recoupling of eNOS was the underlying mechanism of AVE3085 treatment. However, as we did show that AVE3085 reduced myocardial ROS generation, this may suggest AVE3085 prevents oxidation of BH4 by ROS. Finally, it is necessary to investigate the

protective effect of AVE3085 in eNOS knockout mice following TAC to confirm that it is eNOS-dependent effect.

In conclusion, AVE3085 treatment showed pronounced cardioprotective activity in experimental pressure overload remodeling. As we demonstrate, the ameliorating effects of this compound likely results from reversal of eNOS uncoupling and prevention of BH4 oxidation by ROS. Hence, AVE compounds have clear therapeutic potential for the treatment of heart failure, which warrants further study.

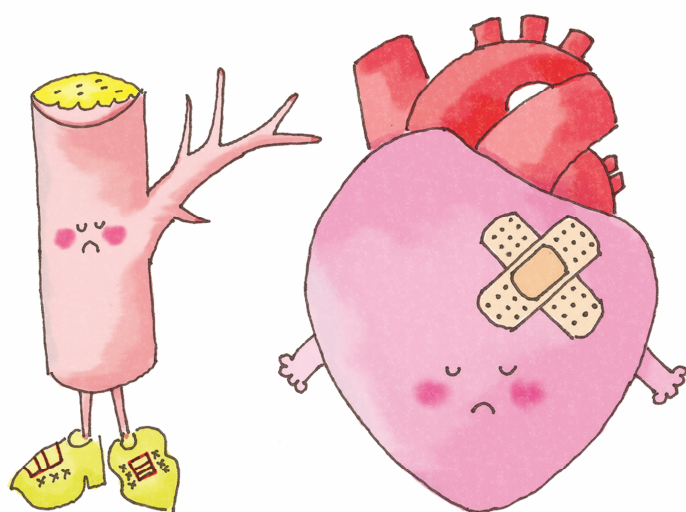
Supplemental Table 1. List of primers used in this study

Gene	Forward primer	Reverse primer
ANP1	TTCCTCGTCTTGGCCTTTTG	CCTCATCTTCTACCGGCATCTTC
BNP1	GGGAGAACACGGCATCATTG	ACAGCACCTTCAGGAGATCCA
aMHC	GGCACAGAAACACCTGAAGA	CATTGGCATGGACAGCATCATC
bMHC	GCCCTTTGACCT CAAGAAAG	CTTCACAGTCACCGTCTTGC
PLB	CCCAGCTAAGCTCCCATAG	AACAGGCAGCCAAATGTGA
GADPH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT

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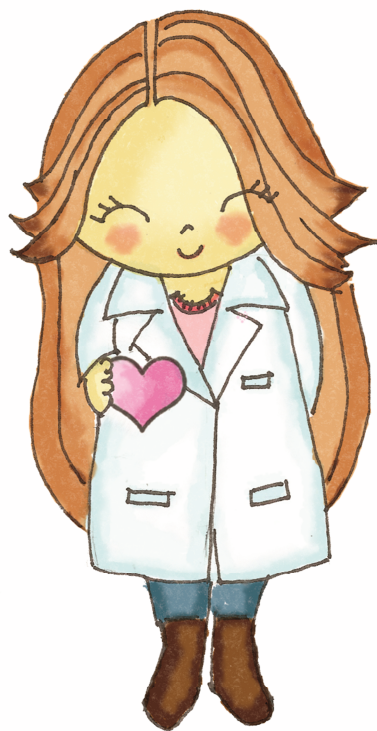


Chapter 6

TNIP2: a novel regulator of inflammatory lymphangiogenesis in human atherosclerosis

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(Submitted)



Chapter 7

General discussion

Main finding of the dissertation

The overall aim of this dissertation was (I) to explore the therapeutic potential of modulating endothelial nitric oxide synthase (eNOS) using AVE3085, an eNOS transcription enhancer, which exerts a reversal eNOS uncoupling, in the setting of cardiac hypertrophy-induced by aortic constriction, (II) to identify specific candidate targets critical in the regulation of human lymphangiogenesis in atherosclerosis, via a systems medicine approach.

The key findings of the work presented in this dissertation are:

1. AVE3085 attenuated cardiac dysfunction and remodeling, through preserved eNOS homodimer instability, decreased myocardial reactive oxygen species (ROS) and reversed the eNOS uncoupling in a mouse model of left ventricular pressure overload (**chapter 5**)
2. Genomics- based methods identify potential novel targets involved in human plaque lymphangiogenesis (**chapter 6**)
3. TNFAIP3 Interacting Protein 2 (TNIP2) is a novel VEGF (vascular endothelial growth factor)-C independent key regulator of plaque lymphangiogenesis in humans, which potentially acts in an inflammation and lipid metabolism dependent manner. The mechanism may involve interferon gamma (IFN γ) and peroxisome proliferator-activated receptor (PPAR)-related lipid and cholesterol metabolism signaling pathway (**chapter 6**).

Correlation of EC function and heart function, focusing on eNOS-NO signaling

The functional integrity of the endothelium is a fundamental element of cardiovascular health. Nitric oxide (NO) generated by eNOS is considered a critical player in maintaining this integrity. Many studies have established that endothelial dysfunction is not only an initiator, but could also be an important factor in the progression of cardiovascular disease. Hereafter, I will discuss the role of endothelial cells (EC) in regulation of cardiovascular system.

The beneficial, regulatory activity of the vascular endothelium in the heart includes vasomotor, hemostatic, antioxidant, and pro- and anti-inflammatory activities^{1, 2}. EC regulate the release of endothelium-induced relaxing factors such NO and endothelium-derived hyperpolarization factor (EDHF), which further stimulates vascular smooth muscle cell relaxation and vasodilation. Likewise, NO in the heart affects the onset of ventricular relaxation, which allows for a precise optimization of cardiac pump function³. In addition, physiologic concentrations of vascular NO as generated by eNOS exerts antioxidant properties⁴⁻⁶, regulates mitochondrial function^{7, 8} and protects against mitochondrial oxidative stress⁹ and thus exerts

cardioprotective effect ¹⁰. Cardiomyocyte paracrine signaling can also regulate EC functions through VEGF-A and angiopoietin (Ang)1 ³. In fact, paracrine cardiomyocyte-to-endothelium signaling and *vice versa* plays a critical role in the vascular adaptations that occur during cardiac hypertrophy. A failure of angiogenesis to match myocyte growth can lead to progressive cardiac dysfunction ¹¹.

Endothelial dysfunction is a hallmark of cardiovascular disease, and it is characterized by impaired endothelial-dependent vasodilatation, increased platelet activation, leukocyte adhesion, and smooth muscle proliferation; and all can be linked to inadequate NO generation ^{12, 13}. Evidence indicates that endothelial dysfunction also results from eNOS dysfunction due to a reduced level of its essential cofactor, tetrahydrobiopterin (BH4). When BH4 levels are depleted, the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, which results in the generation of superoxide anion rather than NO, thus contributing to vascular oxidative stress and endothelial dysfunction ¹⁴. Oxidative stress occurs when excess ROS are generated that cannot be adequately countered by intrinsic antioxidant systems. This plays an important role in the pathophysiology of heart failure ¹⁵. Mechanistically, ROS contribute to the progression of heart failure through stimulating cardiac fibrosis ^{16, 17}, inducing cardiomyocyte apoptosis ¹⁸, modifying proteins central to excitation-contraction coupling ¹⁹ and activating the hypertrophic signaling pathways ¹⁵. In the heart, ROS are generated by several sources, including nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase or xanthine oxidase (XO), eNOS uncoupling, and mitochondria respiration ¹⁵. Given the key role of BH4 in the production of vasoactive substances, such as NO generated by eNOS, it is not surprising that alteration in the bioavailability of BH4 alters cardiovascular function and homeostasis. In **chapter 2**, we highlighted that BH4 acts as a key regulator of eNOS in the setting of cardiovascular health and disease. Importantly, it has been suggested that BH4 deficiency is likely to represent the major cause for eNOS uncoupling ¹². In cardiovascular disease states, there is oxidative degradation of BH4 by ROS. Importantly, the interaction between NO and superoxide results in peroxynitrite generation, the potent oxidant, that enhances BH4 oxidation, leading to eNOS uncoupling. In addition, the exact location of ROS production in the heart remains unclear. eNOS is expressed in vascular endothelium and cardiomyocytes, with the latter representing approximately 20% of total cardiac eNOS ²⁰. This suggests that cardiac EC are the likely locus of the nitro-redox perturbation. Therefore, in **chapter 3**, we discussed how eNOS uncoupling is one of the major underlying causes of endothelial dysfunction. The bioavailability of NO produced by eNOS might be affected at multiple levels, including 1) eNOS mRNA or protein expression, 2) availability of its substrate L-arginine, which might compete with asymmetric dimethylarginine (ADMA) for eNOS binding sites, and 3) availability of its cofactor, BH4, which is determined by a balance of *de novo* synthesis and recycling,

versus BH4 degradation in oxidative stress. Interestingly, BH4 can be regenerated by its oxidized form BH2 via dihydrofolate reductase (DHFR) in the salvage pathway, and can thus maintain eNOS coupling. Inadequate recycling of BH2 to BH4 by DHFR is at least in part, responsible for reduced BH4 levels and the accumulation of BH2, leading to eNOS uncoupling^{12, 21, 22}. Furthermore, in **chapter 4**, we pointed out that modulation of eNOS function is crucial, and can be targeted at multiple levels, including 1) increased eNOS expression and/or activity, 2) augmenting its cofactor BH4 and 3) recoupling the uncoupled eNOS. The small molecule AVE3085 can enhance transcription of eNOS, and interference with eNOS-uncoupling has been suggested. It has been proposed that in pressure overload hypertrophic pathology, oxidative stress is initiated by NADPH oxidase (NOX), causing BH4 oxidation and subsequently resulting in eNOS uncoupling²³ and that eNOS uncoupling is a prominent source of myocardial ROS^{24, 25}. Thus, in **chapter 5**, we tested if AVE3085 can prevent pressure overload-induced left ventricular (LV) hypertrophy. We demonstrated that AVE 3085 attenuated the pathological LV changes associated with pressure overload, in the absence of transcriptionally enhanced eNOS. Although AVE compounds have been shown to increase eNOS transcription and protein expression as discussed in **chapter 4**, we did not observe this beneficial effect. Our findings were similar to a study with porcine coronary arteries incubated with AVE3085, ADMA or AVE3085+ADMA for 1 h which also did not detect the alteration of eNOS protein expression²⁶. The first possible explanation is a compensatory increase in eNOS levels in hypertension; as ROS production is increased, NO production is subsequently decreased²⁷. Therefore, eNOS protein levels may be similar between groups. Alternatively, this compound may not reach the optimal intracellular compartments of cardiac tissue after 7 days. Nevertheless, in **chapter 5**, we are the first to provide evidence for a beneficial effect of AVE3085 treatment, by increasing functional eNOS stability; demonstrated by an increase eNOS dimer to monomer ratio and decreased myocardial ROS generation. AVE3085 attenuated downregulated NOX subunits p22phox and gp91phox²⁸. This may at least, in part, be responsible for protecting BH4 from oxidation. Besides that, AVE3085 exerts pleiotropic effects including lowering blood pressure, improving endothelium-dependent relaxation²⁹, thus these could contribute to improvement of cardiac function if mice following 1-week of TAC.

In summary, endothelial dysfunction is characterized by impaired endothelial NO-dependent vasodilation and is associated with eNOS uncoupling, causing vasoconstriction, leukocyte or platelet activation, and a prothrombotic state. EC dysfunction has been implicated in the underlying pathology of many cardiovascular diseases³⁰. Moreover, endothelial-cardiomyocyte interactions are essential in normal cardiomyocyte function, and in protection from injury. In line with our studies, this suggested that AVE3085 treatment promoted functional eNOS,

reversed the eNOS uncoupling and protected BH4 from oxidation by ROS, subsequently ameliorating endothelial function and thus, may also contribute to improvement of cardiac function. As such, eNOS appears to be a homeostatic regulator of numerous essential cardiovascular functions.

eNOS- NO signaling : protective effects against pressure overload-induced inflammation and fibrosis

In response to injury, the heart initially begins compensatory hypertrophy, however eventually it progresses to maladaptive features, ultimately triggering transition to heart failure³¹. During the progression to heart failure, stressed cardiomyocytes increasingly exhibit contractile dysfunction and cell death³². This process is associated with inflammation and fibrosis³³. Here we discuss main mechanisms underlying the protection of eNOS-NO signaling in pressure overload pathology.

NO is a soluble gas synthesized from L-arginine by NO synthase, with a wide range of biological properties that maintain vascular homeostasis and protect against vascular injury^{4,30}. Under physiological conditions, eNOS is mostly expressed in EC³⁴, and is a major source of NO production in vasculature³⁵. NO dilates blood vessels through activation of soluble guanylyl cyclase (sGC)/ cyclic GMP signaling pathway (cGMP)/ protein kinase G (PKG), resulting in vascular smooth muscle cells (VSMC) relaxation³⁶. NO exerts inhibitory effects on platelet activation and has anti-inflammatory properties³⁵, meanwhile it also protects against EC apoptosis through S-nitrosylation^{37,38}. It is known that inflammatory signaling and macrophage infiltration plays a role in myocardial remodeling and cardiac dysfunction in pressure overloaded hearts^{39,40}. Indeed, inflammation-mediated fibrosis is initiated by macrophage accumulation, associated with fibroblast proliferation and the induction of monocyte chemoattractant protein (MCP)-1 and intercellular adhesion molecule (ICAM)-1 expression in the myocardium. These events are most pronounced three days after TAC^{39,40}. Subsequently, transforming growth factor (TGF)- β expression, which plays a crucial role in the fibrotic process, reached a maximum at day 7⁴⁰. After day 7, concentric left ventricular (LV) hypertrophy developed, associated with reactive fibrosis and myocyte hypertrophy⁴⁰. Taken together, concomitant inductions of MCP-1 and ICAM-1 may indicate their coordinated role in myocardial remodeling in pressure overload hearts, by enhancing macrophage recruitment. Those macrophages in turn produce TGF- β and profibrotic cytokines, leading to accelerated myocardial fibrosis⁴⁰. In line with this view, our results in **chapter 5** demonstrated that AVE3085 ameliorated myocardial fibrosis and myocyte width after TAC. However, the inflammatory process related to TAC was not determined in the present study. Nevertheless, several potential mechanisms could be involved in ameliorating effects

of AVE3085 against TAC-induced inflammation and fibrosis. Indeed, NO decreases MCP-1 expression, and inhibits leukocyte adhesion to the vessel wall, either by interfering with the ability of the leukocyte adhesion molecule CD11/CD18 to form an adhesive bond with the endothelial cell surface, or by suppressing CD11/CD18 expression on leukocytes ⁴¹. Previous studies have demonstrated that one week of TAC did not alter endogenous cardiac BH4 levels ⁴² or affect eNOS activity ²⁵. This suggests that eNOS is functional and can still generate NO production in the presence of sufficient BH4 ^{12, 14}. Our results in **chapter 5** showed that AVE3085 increased eNOS dimer to monomer ratio, as well as L-NAME inhibitable chemiluminescence, could not reduce basal cardiac superoxide generation. This indicates the enhanced capacity of NO generated by functional dimeric eNOS, as well as restored NO bioavailability. Thus, the perivascular macrophage accumulation observed shortly after TAC ^{39, 40} may be prevented by AVE3085 mediated eNOS-generated NO production in our study. Besides this, ROS can activate proinflammatory mediator cascades, and is involved in the development of interstitial cardiac fibrosis ⁴³. AVE3085 inhibited ROS production, thus it might also reduce inflammatory cytokine production and fibrosis in TAC hearts. In addition, recent evidence shows BH4 involvement in inflammatory cell signaling ⁴⁴ and inhibition of macrophage infiltration and blocked inflammatory signaling by oral exogenous BH4 three days after TAC in mice ⁴⁵. We showed in **chapter 5** that AVE3085 protected BH4 oxidation and tended to induce mRNA expression of the BH4-generating enzyme GCH1. Since cardiac BH4 levels were not altered one week after TAC ⁴², the sufficient endogenous BH4 availability combined with the BH4 levels that were restored by AVE3085, may appear similar to the effect of exogenous BH4 ⁴⁵. AVE3085 might have attenuated inflammatory signaling through restored (or even enhanced) BH4 levels and subsequently BH4 prevented TAC-induced inflammation and fibrosis. The exact role of AVE3085 in inflammation-associated pressure overload remains to be elucidated.

As discussed in **chapter 4**, AVE3085 exerts pleiotropic effects, including anti-fibrotic properties, through downregulation of TGF- β and connective tissue growth factor (CTGF) in experimental diastolic heart failure ²⁸, and inhibition of Smad (mothers against decapentaplegic) 2/3 signaling in mice following 4 weeks of TAC ⁴⁶; thus it protects against cardiac fibrosis. Moreover, AVE3085 attenuated adhesion molecules ICAM-1 and VCAM-1 in experimental diabetes ⁴⁷. Together, these findings show that AVE3085 may directly inhibit inflammation-mediated fibrosis caused by TAC. In summary, based on our findings, possible mechanisms of AVE3085 mediated TAC-induced inflammatory response may result from (1) indirect effects, secondary to inhibition of macrophage infiltration and inflammatory signaling mediated by NO and/or BH4, or (2) pleiotropic effects of AVE3085 on inflammation and fibrosis.

When eNOS is healthy, it produces NO which acts as protector of cardiovascular health. When eNOS becomes dysfunctional, it shifts towards ROS generation and acts as pro-oxidant, rather than an antioxidant enzyme. Below, we discuss how dysregulation of eNOS can contribute to inflammation and progression of cardiovascular disease.

Effect eNOS on inflammation

Under physiological condition, eNOS produces a low basal amount of NO which exerts anti-inflammatory properties, and is responsible for cardiovascular homeostasis through regulating the interaction between the endothelium and inflammatory cells ⁴⁸.

A hallmark of inflammation is increased vascular permeability, during which inflammatory cells migrate and interact with EC ⁴⁸. Upon activation, the endothelium releases inflammatory cytokines as well as adhesion molecules, facilitating the recruitment and attachment of circulating leukocytes to the vessel wall ⁴⁹. During inflammation, local agonists (e.g. shear stress and VEGF-A) that act on EC can influence eNOS activity through post-translational modifications ^{48, 50}. It is known that phosphorylation of eNOS at serine (Ser)-1177 enhances eNOS activity ^{51, 52}, and directly regulates NO and superoxide generation; the latter's activity depends on eNOS coupling status (i.e. when eNOS is uncoupled, phosphorylation of Ser-1177 increases eNOS-dependent superoxide generation) ⁵³. NO reacts with superoxide and forms peroxynitrite, a potent proinflammatory and apoptotic agent ⁵⁴, indicating that phosphorylation of eNOS can modulate peroxynitrite generation. This suggests that under pathologic conditions where eNOS is often uncoupled ⁵⁵, activation of eNOS activity can exacerbate inflammatory processes.

VEGF-A is crucially involved in several chronic inflammatory disorders, in which it not only promotes pathologic angiogenesis but directly fosters inflammation ^{56, 57}. It has been suggested that eNOS may contribute to the inflammatory process indirectly through the stimulation of VEGF-A-induced permeability, as well as VEGF-A-dependent-adhesion molecule expression. Indeed, VEGF-A upregulates the expression and activity of eNOS ^{58, 59}, which in turn causes eNOS-derived NO to modulate both VEGF-A- induced angiogenesis and permeability ⁶⁰.

The proinflammatory effect of eNOS-derived NO occurs, at least in part, via a modulation of the activity of the transcription factor nuclear factor kappa beta (NFkB) ⁶¹. NFkB has been shown to be a key regulator of proinflammatory genes, including cytokines, chemokines, and adhesion molecules ⁶². It has also been demonstrated that laminar shear stress activates NFkB, which in turn increases eNOS transcription

and expression, and thus enhances NO production. Subsequently, this NO inhibits NFκB activation in a negative feedback loop, resulting in the termination of eNOS transcription^{63, 64}. However, increased oxidative stress accelerates NO degradation through its reaction with superoxide, in turn peroxynitrite causes eNOS uncoupling through oxidation of BH4³⁴. The consequences of eNOS uncoupling are both reduced synthesis and bioavailability of NO⁴¹, which can cause substantial activation of NFκB and leads to endothelial inflammation⁶⁴.

Interestingly, as discussed in **chapter 3**, although many studies have shown that eNOS overexpression generates cardiovascular protective effects by increasing endothelium-derived NO^{65, 66}, the expression of eNOS protein in endothelial dysfunction remains unchanged, or may, in some cases, even be increased⁶⁷. In pressure overload pathology, lack of eNOS ameliorated^{24, 68} TAC-induced cardiac remodeling and dysfunction⁶⁸, whereas eNOS overexpression exacerbated this. The latter was associated with increased myocardial ROS production and enhanced eNOS uncoupling⁶⁸. Because uncoupled eNOS not only generates NO, but also generates ROS, one should be aware that increased eNOS transcription, translation or activity does not guarantee improved endothelial function⁶⁹. Hence, pharmacological approaches to preserve or restore eNOS functionality and prevent eNOS uncoupling are warranted. On the basis of the pathophysiology mentioned above, as discussed in **chapter 2-4** there are several possible approaches to restore eNOS functionality (ie, recouple eNOS). This further highlighted that indeed, elevated eNOS expression without further increase in BH4 levels, results in eNOS uncoupling because of the enzyme cofactor imbalance⁷⁰. **In chapter 5**, we demonstrated that treatment of AVE3085 1 week after TAC prevented BH4 oxidation rather than increased its synthesis. The long term effects of AVE3085 on BH4 levels in TAC mice, however, is not yet known.

In summary, eNOS-derived NO has an important role in preserving homeostasis in the interaction between the endothelium and inflammatory cells in basal physiological conditions. Post-translational modifications impact eNOS activity and can contribute to inflammatory process. Pharmacological approaches that increase eNOS protein levels are only beneficial when the produced eNOS is functional, and strategies to preserve or restore eNOS functionality and prevent eNOS uncoupling are thus warranted.

Opposite effects of angiogenesis in the heart versus atherosclerosis

The role of angiogenesis in cardiovascular diseases depends on the pathophysiological context, and seems opposing in cardiac and vascular pathology. Vascular insufficiency occurs in cardiac hypertrophy^{71, 72}, myocardial infarction and coronary ischemia⁷³. Patients with cardiomyopathy displayed decreased coronary capillary density in histological sections⁷⁴. In contrast, excessive plaque angiogenesis is considered detrimental⁷⁵, and neovessel formation is associated with disease progression and destabilization⁷⁶.

In physiological cardiac hypertrophy, increased coronary angiogenesis is associated with preserved contractile function, whereas pathological cardiac hypertrophy (reduced contractility) is accompanied by impaired coronary angiogenesis^{77, 78}. In experimental pressure overload-induced cardiac hypertrophy, blocking VEGF-A-dependent angiogenesis accelerated the transition from compensated to decompensated heart failure⁷⁹, whereas activation of angiogenesis using adenovirus-mediated expression of VEGF-A and Ang1 enhanced preserved contractile function⁸⁰. Interestingly, pressure overload initially promoted cardiac angiogenesis through hypoxia-inducible factor-1 (HIF-1)-dependent angiogenic response. However, prolonged pressured overloaded resulted in reduced HIF-1 activity, was associated with impaired cardiac angiogenesis, despite persistent myocardial hypoxia in the hypertrophied hearts. This suggested that mismatch of angiogenesis in hypertrophied hearts exacerbated myocardial hypoxia and cardiac dysfunction. Taken together, angiogenesis is essential for adaptive cardiac growth, and that disruption of angiogenesis signaling impairs heart function and contributes to the progression from adaptive cardiac hypertrophy to heart failure^{77, 79}.

Plaque angiogenesis is the response to hypoxic conditions⁸¹. Plaque hypoxia predominantly occurs due to increasing oxygen demand in the inflammatory microenvironment, although plaque thickness also contributes to reduction of oxygen supply⁷⁶. In addition, hypoxia-induced HIF-1-dependent proangiogenic gene expression has been implicated as an important mechanism responsible for plaque angiogenesis. Angiogenesis may be important for plaque pathogenesis during the initial stages of lesion growth, though it is also plausible that angiogenic stimuli may have a destabilizing role in the mature plaque⁷³. However, neovessels, in particular those in the plaque shoulder region, appear to be immature and highly susceptible to leakage, and can cause intraplaque hemorrhage⁸². Ruptured plaques of patients that manifested atherosclerosis displayed increased microvessel density in ruptured plaques, as well as in the intraplaque hemorrhage, suggesting a contributory role for neovascularization in the process of plaque rupture⁸³. Moreover, vasa vasorum (VV) density is correlated with disease severity^{76, 84}, as well as with inflammatory cell

density in the plaque lesions⁸⁵. Taken together, plaque angiogenesis is a physiological adaptation, but the associated proinflammatory and proatherogenic effects are deleterious for plaque vulnerability.

In summary, angiogenesis is beneficial in cardiac hypertrophy and ischemia by increasing blood perfusion in ischemic tissues and improving cardiac function. On the other hand, angiogenesis is detrimental in atherosclerosis by promoting disease progression and is considered as a risk factor for plaque rupture.

Presence and functions of lymphatics in plaque

Lymphangiogenesis and angiogenesis are tightly intertwined processes, which are orchestrated by similar regulatory cues⁸⁶. Moreover, leakage of (newly formed) dysfunctional vessels will lead to interstitial pressure buildup, which in turn will trigger lymphangiogenic responses, in order to drain redundant fluids and restore interstitial pressure⁸⁷. Therefore it is not unsurprising that besides angiogenesis, also lymphangiogenesis is instrumental in cardiovascular physiology and pathophysiology. Increased lymphatic density is associated with cardiovascular diseases, including progressive atherosclerosis, acute and chronic myocardial ischemia, myocarditis, endocarditis and cardiac hypertrophy^{88, 89}. In animal studies, lymphatics were observed in the adventitial and periadventitial regions of the arterial wall⁹⁰⁻⁹². In human atherosclerotic internal carotid artery, iliac artery and aorta lesions, lymphatic vessels are detected in the adventitia. In fact, the adventitial lymphatic density was positively correlated with atherosclerosis severity, as assessed by intimal thickness^{93, 94}, suggesting a link between lymphatics and disease progression. Kholova et al.⁸⁸ observed lymphatic vessels in fibro-calcified and fibrous atherosclerotic lesions. They reported profound lymphatic growth in adventitia, media and intima of atherosclerotic sections; the latter two were present only in progressive lesions. In plaque lesions, lymphatics were present in plaque shoulder and cholesterol crystal-rich area in plaques and associated with lymphatic remodeling, including collapsed and dilated lymphatic vessels. In agreement with this observation, in **chapter 6**, we observed podoplanin⁺ lymphatic vessels were abundantly present in the adventitia and within atherosclerotic lesion such as in cholesterol crystal-rich area of ruptured segments. Importantly, we observed podoplanin⁺ lymphatic vessels in the intima of advanced ruptured plaque lesions. This observation was consistent with previous studies observing lymphatic vessel development in the intima and was associated with areas rich in extracellular matrix as well as lipid core region^{88, 95}. Conversely, other studies observed no⁹⁶ or rare lymphatic vessels and those lymphatic vessels mostly presented in the adventitia of human atherosclerotic vessels⁹⁷. Together, lymphatic vessels have been readily identified in atherosclerotic and non-atherosclerotic arterial

clinical samples in most, but not in all studies. This controversial result warrants further investigation.

Lymphangiogenesis driving factors in plaque

As mentioned, given the association between progressive atherosclerosis and lymphangiogenesis, it was postulated that lymphangiogenesis exacerbated atherosclerosis, and that intervention in lymphangiogenesis might be beneficial. In line with this view, Xu et al.⁹⁸ hypothesized that in the atherosclerotic artery, adventitial lymphatics facilitate the delivery of inflammatory cells draining lymph nodes and lymphoid tissue, as well as their activation therein. The activated cells and derived cytokines will be transported via blood vessels to the plaque, causing intimal hyperplasia and vascular remodeling. Conversely, it has been suggested that the imbalance of angiogenesis and lymphangiogenesis in plaque may result in fluid buildup in atherosclerotic plaque and that a loss of lymphatics accelerates plaque progression⁹⁷. In particular, recent evidence from mouse models support an essential contribution of lymphangiogenesis to the regression of atherosclerosis. Indeed, studies have revealed that lymphatics are critical for immune response and cholesterol metabolism in atherosclerosis. Moreover, hypercholesterolemic mice displayed lymphatic structural defects⁹⁹. Lymphatic dysfunction may already occur before atherosclerosis onset¹⁰⁰. In addition, pro-lymphangiogenic interventions reduced atherosclerosis, while anti-lymphangiogenic intervention enhanced plaque burden^{101, 102}.

Role of lymphatics in atherosclerosis

The lymphatics function I) as a route for migratory cells trafficking between plaque and secondary lymphoid organs¹⁰³, and II) as conduit for drainage of interstitial macromolecules. The former function is evidenced by regulation of local T cell content by adventitial lymphatics¹⁰². Indeed, an impaired capacity of monocyte-derived cells to emigrate out of the inflamed site through nearby lymphatic vessels is associated with atherosclerosis progression due to failure to resolve inflammation^{103, 104}. Moreover, surgical interruption of lymph drainage or blockade of VEGF-C/D-dependent lymphangiogenesis in collar-induced plaque in apoE^{-/-} mice resulted in exacerbated atherosclerotic plaque burden and increased intimal and adventitial CD3⁺ T cell content¹⁰². Together, this suggests that lymphatic dysfunction may stimulate atherogenesis through enhanced inflammation, and hence contribute to atherosclerosis progression.

The second function of lymphatics is drainage of interstitial macromolecules back into the circulation. As lymph contains 30% more high-density lipoprotein (HDL) cholesterol than blood, this suggests a key role of lymphatic circulation in regulating

reverse cholesterol transport (RCT) ¹⁰⁵. Surgical or genetic-induced lymphatic disruption in mice models emphasized the important role of lymphatic vessels in facilitating cholesterol removal from skin and transplanted aortic vessel ^{101, 106}. Importantly, transplantation of pre-established atherosclerotic aortas, *ex vivo* incubated with stable isotope labeled cholesterol, to apoE^{-/-} recipient mice with impaired lymphangiogenesis (anti-VEGFR-3 treatment), caused cholesterol accumulation in the transplanted aortic plaque tissue ¹⁰¹, and highlighted the essential role of lymphatics in removing cholesterol from aortic plaques. Furthermore, lymphatic insufficient transgenic mice displayed increased cholesterol and triglyceride levels in plasma, and exhibited accelerated plaque formation, and that atheroma formation was inversely associated with abundance of adventitial lymphatics in aorta ¹⁰⁷. This highlights the critical role of lymphatics in cholesterol metabolism, and indirectly supports the role of lymphatics in the removal of cholesterol from the atherosclerotic vessel. Moreover, Milasan et al. ¹⁰⁰ demonstrated in atherosclerotic low density lipoprotein receptor –deficient mice expressing the human apolipoprotein B100 transgene (LDLR^{-/-} /hApoB100^{+/+}) that 3-month old mice displayed lymphatic dysfunction before atherosclerosis onset and that lymphatic dysfunction was associated with a defect of collecting lymphatic vessels in LDLR-dependent manner.

In **chapter 6**, we sought to identify novel players in pathologic lymphangiogenesis in human atherosclerosis. We employed a bioinformatics approach, weighted gene coexpression network analysis (WGCNA), to identify networks and candidate genes relevant to plaque lymphangiogenesis in humans. In this study, we correlated phenotypic traits i.e. D2-40⁺ lymphatic vessel density (LVD) in human atherosclerotic tissue, with transcription profiling derived from the same tissue samples. Using this approach we identified modules showing highest correlation to LVD. Subsequently, we could select potential candidate genes as well as transcription factor binding sites (TFBS), miRNAs, and potential pathways which may be involved in regulation of plaque lymphangiogenesis.

Taken together, lymphatic dysfunction and impaired lymphatic drainage accelerate plaque progression. Lymphatic vessels could be expected to have a protective effect against atherosclerosis by affecting both RCT and the local inflammatory milieu.

Dual signaling cascades stimulate lymph- and angiogenesis

The formation of lymphangiogenesis is a complex process, which has similarities with angiogenesis. Lymphangiogenesis and angiogenesis share regulatory mechanisms and often coexist in atherosclerotic lesions ⁹⁵. The adventitial lymphatic absorbing networks consist of large and sparsely distributed capillary structure, which form a wrapping plexus around large caliber arteries (periarterial lymphatics) ⁹⁰. This

anatomical arrangement of the lymphatics within the arterial wall appeared to be consistent with arterial branches in the viscera, suggesting the role of lymphatics as an important drainage route for the arterial interstitium⁹⁰.

Mechanistically, several factors that enhance lymphangiogenesis also promote angiogenesis⁸⁶, including VEGF-C/D/VEGFR-3, VEGF-A/VEGFR-1/2 and Ang1/Ang2 signaling. The first signaling axis is well established to promote lymphangiogenesis¹⁰⁸, however, mature VEGF-C and VEGF-D which are generated by proteolytic processing, can bind and activate VEGFR-2^{109, 110}, and thus induce angiogenesis¹¹¹. Interestingly, it has been demonstrated that VEGFR-2, but not VEGFR-3, is expressed in the vessel wall at every stage of atherosclerosis¹¹². In addition, VEGFR-2 can bind to and reduce VEGF-C activity, and subsequently cause inhibition of lymphangiogenesis¹¹³. This may suggest that the interaction between VEGF-C/ VEGFR-2 may contribute to imbalance between lymphangiogenesis and angiogenesis during atherosclerosis progression.

The second dual signaling pathway involves VEGF-A, for which, signaling through VEGFR-2 is the major angiogenesis pathway⁷⁶. However, VEGF-A also induces a lymphangiogenic response¹¹⁴, which is mediated mainly through VEGFR-2^{115, 116}. Interestingly, transduction of murine VEGF-A to the ear skin of mice induced abnormal lymphangiogenesis, characterized by enlarged lymphatic vessels, incompetent valves, and delayed lymph clearance, suggesting that overexpressed VEGF-A might result in development of abnormal lymphatics¹¹⁴. Thus, high expression of VEGF-A in atherosclerotic lesions^{117, 118} may at least in part contribute to lymphatic dysfunction, leading to insufficient lymphatic drainage and subsequently exacerbate atherosclerosis formation.

The third overlapping signaling pathway involves Ang1 and Ang2. These two proteins have distinct functions in the blood vascular system, but redundant roles in lymphatic development¹¹⁹. In blood vessels, Ang1 and Ang2 can be pro-or anti-angiogenic, owing to their respective agonist and antagonist signaling action through the Tie2 receptor¹²⁰. In lymphatics, Ang2 is a prerequisite for lymphatic remodeling, stabilization, maturation and development^{121, 122}, specifically in regulating of lymphatic valve morphogenesis¹²³. Ang2 deficient mice exhibited lymphatic defects, which could be rescued by Ang1 knock-in in the Ang2 genetic locus, suggesting a redundant role in lymphatic development, where either ligand can act as a Tie2 receptor agonist¹²². Ang2 has a stimulatory role in various processes associated with vulnerable plaques such as increased inflammation, plaque vascularity, and microvascular leakage¹²⁴. Interestingly, Ang2 partially rescued the reduced lymphangiogenesis when VEGF-C/VEGFR-3 signaling was blocked, suggesting a role of lymphangiogenic factor¹²³. The exact role of Ang2 in plaque lymphangiogenesis remains to be elucidated.

In **chapter 6**, we identified novel lymphatic regulators in atherosclerosis. Our findings demonstrated that TNIP2 was associated with human plaque lymphatics, and was critical for lymphangiogenesis *in vitro*. TNIP2 expression was induced by inflammatory and lipid stimuli. TNIP2-silenced lymphatic endothelial cells (LEC) showed profound downregulation of LEC-specific genes and lymphangiogenic factors and impaired LEC proliferation, migration and differentiation. Further support for the involvement of TNIP2 in lymphangiogenic regulation was derived from the reciprocal effects of TNIP2 and the lymphangiogenic master regulator PROX1. We found inverse regulatory roles of TNIP2 and PROX1 in lymphangiogenesis *in vitro*, where TNIP2 knockdown appears to dampen PROX1 target gene expression, and thus LEC differentiation. It is also of note that other studies reported an interaction of TNIP2 with Tie2. This interaction depends on Tie2 autophosphorylation and is further enhanced by Ang1 stimulation ¹²⁵. This suggests that TNIP2 may also regulate lymphatic development through Ang1/Tie2 signaling.

Together, the formation of lymph- and angiogenic vessels is co-dependent, and mediated by shared signaling pathways such as VEGF-C/D/VEGFR-3, VEGF-A and Ang1/Ang2 signaling. TNIP2 may act as a novel regulator of lymphangiogenesis.

Interventions in lymphangiogenesis to ameliorate disease?

Emerging evidence has suggested that enhancement of lymphangiogenesis and lymphatic function in plaque may represent an effective therapeutic approach for inflammatory diseases ¹²⁶, including atherosclerosis ^{95, 100, 101, 107}. To date, there are limited data on whether pro-lymphangiogenic targets could alter the risk for atherosclerosis ¹²⁷. Given that there is still much unknown regarding the role of lymphangiogenesis in atherosclerosis, the actual effects of therapeutic administration are unclear. While pro-lymphangiogenic therapy seems to be an attractive strategy for the treatment atherosclerosis, excess activation of lymphangiogenesis may result in increased exposure of lymph nodes to inflammatory mediators and promote the development of metastasis ¹²⁸. Therefore, the safety and efficacy of the lymphangiogenic therapy must be carefully evaluated within the context of each disorder and the desired therapeutic goals ¹²⁷.

In **chapter 6**, we have demonstrated that TNIP2, an inhibitor of NFκB, may positively regulate lymphangiogenesis in chronic inflammatory conditions in plaque. TNIP2 may be a potent target to approach in inhibiting atherosclerosis development, as it inhibits NFκB, a master regulator of inflammatory and atherogenic genes ¹²⁹. We found that TNIP2 was upregulated in ruptured plaque segments in comparison to stable plaque segments. We further demonstrated that TNIP2 negatively regulated IFNγ a key proinflammatory cytokine in atherogenesis ¹³⁰. Moreover, IFNγ was seen to

exert anti-lymphangiogenic properties in murine, porcine and human LEC^{131, 132} which is compatible with our findings. TNIP2 also positively regulated PPAR γ activity, although there is little evidence for direct regulatory effect of PPAR γ on lymphangiogenesis. Interestingly, PROX1 interacts directly with PPAR γ and induces the downregulation of IFN γ in T cells, suggesting PROX1 acts as a co-repressor for IFN γ ¹³³. Moreover, PPAR γ can antagonize NF κ B signaling-mediated proinflammatory pathways in macrophages¹³⁴. Together, this may suggested the tight collaboration between TNIP2, PROX1 and PPAR γ in mediating proinflammatory pathways related to lymphangiogenesis *in vitro*. It has been demonstrated that activation of NF κ B by inflammatory stimuli (i.e. IL-3 and LPS) elevated PROX1 and VEGFR-3 expression in cultured LEC, resulting in increased proliferation and migration; thus suggesting a role for inflammation-induced lymphangiogenesis¹³⁵. Interestingly, we demonstrated in **chapter 6** that treatment of LEC with LPS also upregulated TNIP2 mRNA expression. As mentioned above, we found that TNIP2 might induce PPAR-related lipid and cholesterol metabolism signaling. Together, this may indicate that TNIP2 cooperates with PPAR γ to play a negative feedback role in the regulation of NF κ B-mediated inflammatory lymphangiogenesis *in vitro*.

In conclusion, we propose that TNIP2 could be a potent target in inhibiting atherosclerosis development; by inhibiting NF κ B dependent inflammation and IFN γ dependent lymphangiogenesis and enhancing PPAR- dependent lipid fluxes *in vitro*. Future delineation of these mechanisms might identify targets for therapeutic lymphangiogenesis in atherosclerosis.

The therapeutic potential of intervention in lymphangiogenesis for atherosclerosis is not well studied. One may also question that if increased lymphatic density is associated with plaque progression and is consistently found in advanced ruptured plaque, if lymphangiogenesis may thus even promote atherogenesis. However, evidence from *in vivo* studies have suggested that interrupted lymphatic drainage exacerbated atherosclerosis development, whereas inversely, promotion of lymphangiogenesis ameliorated atherosclerosis development^{100-103, 106, 107}. Indeed, recent evidence demonstrated that VEGF-C 152s administration in this mouse model resulted in increased lymphatic vessel density in the adventitia of the aortic sinus and improved lymphatic cellular transport¹⁰⁰. However, targeting VEGF-C remains somewhat controversial, as Ad-VEGF-C or AAV-VEGF-C treatment caused blood vascular changes, including enlargement, tortuosity and leakiness via interaction with VEGFR-2¹³⁶. Angiogenesis promotes plaque progression and lesion instability⁷⁶ thereby raising concerns about VEGF-C capability to cause off-target proangiogenic effects. In addition, recent evidence has reported potential pro-lymphangiogenic agents, such as the FDA-approved 9-cis retinoic acid (9-cis RA; alitretinoin)^{137, 138} or cholesterol-lowering compounds such as ezetimibe and proprotein convertase

subtilisin/kexin type 9 (PCSK9) inhibitor, which may show pleiotropic roles in improvement of lymphatic functions, as it is documented in experimental atherosclerosis^{100, 106}. However, as we show in **chapter 6**, TNIP2, being a specific regulator of pathologic lymphangiogenesis during atherosclerosis development and progression, would represent a much more suitable clinical target. Together, although lymphatics likely are important, the causal role and therapeutic options for plaque lymphangiogenesis remain underexplored.

The suitability of animal models of atherosclerosis for studying plaque lymphangiogenesis.

Zebrafish have gained interest for studying dyslipidemia, and genes related to atherosclerosis¹³⁹. Several genes involved in lipid and lipoprotein metabolism including major classes of apolipoproteins, CETP, PPARs, LXLR and LDLR are conserved from zebrafish to human¹⁴⁰⁻¹⁴². In addition, zebrafish develop only the early stages of atherosclerosis by interventions¹⁴³⁻¹⁴⁷. Zebrafish are however, suitable for studying inflammatory lymphangiogenesis¹⁴⁸, investigating novel anti-lymphatic compounds¹⁴⁹ and screening to identify candidate genes or novel pathways, leading to discoveries such as the novel mammalian lymphatic gene CCBE1¹⁵⁰. In **chapter 6**, we have demonstrated in zebrafish that *tnip2* deficient morphants had an increased number of venous intersegmental vessels (vISVs), parachordal lymphangioblasts (PLs) and thoracic ducts (TD). In the secondary wave of zebrafish angiogenesis, half of the sprouts that directly bud from the posterior cardinal vein (PCV) reconnect with and convert primary arterial sprout-derived ISVs into vISVs. The other half do not connect, continue to migrate dorsally and constitute so-called parachordal lymphangioblasts (PLs), that align along the horizontal myoseptum, after which they migrate dorsally or ventrally (then referred to as LEC) at 3 days post-fertilization (dpf)^{150, 151}. Since we found increased vISVs, we expected to see less PLs. Surprisingly, we also observed more PLs. This could only mean that more venous sprouts have emerged from the PCV, indicative of enhanced sprouting activity of *tnip2* in venous endothelial cells (VEC). It is well documented that in zebrafish, *vegf-c* induced VEC proliferation in the PCV^{152, 153}. *ccbe1* and *vegf-c* are required for the sprouting of venous precursors and PLs from the PCV during secondary angiogenesis^{150, 154}. Indeed, we found that TNIP2 silencing in LEC *in vitro* resulted in upregulated CCBE1 and VEGF-C, but downregulated PROX1 and SOX18 mRNA expression. This may indicate that the upregulation of *vegf-c* and *ccbe1* expression may contribute to the increased vISVs, PLs and TD in *tnip2* deficient morphants. In contrast, *prox1*, *coup-TFII* and *sox18* are not essential for lymphatic development in zebrafish¹⁵⁵, this suggests another mode of lymphatic commitment¹⁵⁶. This therefore also raises a note of caution regarding the interpretation of zebrafish studies. Alternatively, it is possible that TNIP2 effects are more prominent in adult lymphangiogenesis under pathological conditions. Together,

this might also explain that *tnip2* deficient morphants did not exhibit pronounced lymphangiogenesis defects. Nevertheless, our finding suggested that TNIP2 negatively regulates sprouting activity of endothelial cells *in vivo*, this seems to be specific for venous/lymphatic endothelial cells.

To date, most experimental lymphangiogenesis-associated atherosclerosis studies have utilized a combination of atherosclerotic mouse models with genetic and/or surgical interventions to induce lymphatic insufficiency. This raises the question whether the methods or results can be translated to human atherosclerosis. Wildtype mice are resistant to atherosclerosis because they exhibit high plasma HDL and low LDL and VLDL distribution, which is opposite to human lipoprotein profiles¹⁵⁷⁻¹⁵⁹. Nevertheless, mice models are suitable to study mechanisms, but one should be aware of differences hampering translation to human disease.

Unlike mice and rabbits, pigs develop spontaneous atherosclerosis upon feeding, and the development of plaques can be induced by intervention¹⁶⁰. In addition, their plaque lesions closely mimic advanced human plaques¹⁶¹. A porcine disease model thus seems most suitable for the study of atherosclerosis related diagnostic and therapeutic interventions¹⁶²; pig models of coronary atherosclerosis allow investigation of the impact of adventitial neovascularization by coronary imaging technologies^{162, 163}, and therefore may be applicable to adventitial lymphangiogenesis research. Therapeutic intervention targeting pro-lymphangiogenic factors have been investigated in lymphedema pigs¹⁶⁴⁻¹⁶⁶, which further suggested that similar strategies could be employed to investigate atherosclerosis¹²⁷. As such, a new candidate or therapy focused on lymphangiogenesis should be tested in a pig model before venturing to human disease.

Conclusions and Perspectives

In this thesis we addressed the role of eNOS and therapeutic possibilities in experimental pressure overload-induced cardiac hypertrophy in mice. eNOS-derived NO is crucial in maintaining cardiovascular homeostasis. In pressure overload-induced cardiac hypertrophy, eNOS uncoupling is a major source of myocardial ROS, which then contribute to dilatory remodeling and cardiac dysfunction¹⁶⁷. Improvement of eNOS activity by stabilizing eNOS function and suppressing eNOS-derived ROS production is thus a promising therapeutic target for cardiovascular diseases. Compounds that increase eNOS expression and/or activity are only beneficial when accompanied by eNOS functionality³⁵. Elevated eNOS expression without further increase in BH4 levels, generally results in eNOS uncoupling, because of the enzyme cofactor imbalance. We demonstrated that the eNOS enhancer AVE3085 also restored cardiac BH4 levels, reversing eNOS uncoupling in a mouse model of left ventricular pressure overload. Our study emphasizes the importance of eNOS as a

regulator in nitroso-redox balance and as a therapeutic target in left ventricular hypertrophy induced by aortic stenosis, suggesting that eNOS enhancement may benefit heart failure patients. Despite the promising results in our *in vivo* experiments, the long-term therapeutic benefit of AVE3085 is not yet known. Further studies are required to better understand how modulation of nitroso-redox balance can be optimized and deployed to attenuate cardiac remodeling and dysfunction induced by aortic stenosis.

In the second part of my thesis, we have investigated another process in homeostatic fluid balance: lymphangiogenesis. Although recent studies have suggested the protective roles of atherosclerotic plaque lymphatics, the process of pathological lymphangiogenesis in the plaque is, however, not yet completely understood. Herein we provided a proof of concept that candidate genes correlating with plaque lymphangiogenesis, as identified by weighted coexpression analysis, do indeed control this process. Our study highlights TNIP2 as a novel gene regulator of plaque lymphangiogenesis, potentially through mechanisms related to inflammation and lipid metabolism. Further studies are required to dissect molecular mechanism of TNIP2-dependent lymphangiogenesis and to functionally validate our findings in other animal models.

In conclusion, we have provided evidence of molecular regulation of cardiovascular disease, focusing on eNOS and TNIP2. The next challenge is to integrate these findings and translate them to clinical utility for cardiovascular disease prevention and/or treatment.

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Summary

Summary

Cardiovascular disease (CVD) remains the most common cause of death in Europe and worldwide accounting for 31% of all deaths. Importantly, atherosclerosis is by far the most important cause of CVD, accounting for 80% atherosclerosis of CVD worldwide. Atherosclerosis is a lipid-driven chronic inflammatory disease, leading to the formation of plaques at vital segments of the arterial tree. Atherosclerotic plaques are deposits of cholesterol, lipids and cellular debris in the artery wall, resulting in narrowing of the larger arteries. Initial atherosclerotic lesions are characterized by subendothelial accumulation of macrophages and macrophage-derived foam cells. In progressive stages of disease development, medial smooth muscle cells will proliferate, migrate into the intima and produce collagen, forming a fibrous cap that covers the plaque (i.e. stable lesion). The vulnerable plaque is characterized by an extensive lipid core in the central atheroma, a high level of inflammation and a thin and inflamed fibrous cap covering the large necrotic core (thin-cap fibroatheromas; TFCA). In the final disease stage this vulnerable plaque will rupture leading to thrombus formation which can occlude the lumen causing distal ischemia and acute cardiovascular events (e.g. myocardial infarction (MI), stroke).

Endothelial cells play an important role in maintaining cardiovascular homeostasis. In this thesis we have focused on the molecular regulators thereof. The aim of this thesis is two-fold: 1) to study endothelial cells (EC) dysfunction mediated by impaired NO in heart failure, and 2) to define new molecular regulators of lymphatic EC (LEC) in atherosclerosis.

The first part of the thesis involves a study of endothelial dysfunction in the heart and vessels, which is often characterized by impaired NO bioavailability, leading to vasoconstriction, coagulation and inflammation. When chronic, this can lead to several pathological conditions, including MI, hypertension and atherosclerosis. In this dissertation it is hypothesized that modulation of endothelial nitric oxide synthase (eNOS) can protect against pressure overload-induced left ventricular hypertrophy.

In the first part of this thesis (**chapter 2-5**) are focused on the molecular mechanism of eNOS and its essential cofactor tetrahydrobiopterin (BH4), as well as potential therapeutic interventions to modulate eNOS in the pathogenesis of myocardial and endothelial dysfunction. As discussed in **chapter 2**, BH4 is a critical regulator of cardiovascular homeostasis, and substantial evidence implicates BH4 as a key regulator of eNOS in the setting of cardiovascular disease. Strategies to maintain BH4 bioavailability may be achieved by 1) enhancing the regeneration of BH4 from the inactive form BH2, 2) chemically stabilizing BH4, and 3) reducing oxidative degradation. Indeed, eNOS-generated NO has a crucial role in the regulation of endothelial function. **Chaper3** provides an extensive overview of the important role

of eNOS-uncoupling in the pathogenesis of endothelial dysfunction. Evidence suggests that modulation of eNOS by stabilizing eNOS function, and suppressing eNOS-derived ROS is a promising therapeutic target for endothelial dysfunction. Subsequently, in **chapter 4**, two, novel pharmacologic small molecule compounds that transcriptionally enhance eNOS gene expression, AVE9488 and AVE3085, are discussed. These compounds are designed to increase eNOS transcription and enhance NO signaling and bioavailability. Interestingly, AVE have been shown to also increase BH4 bioavailability reversing eNOS uncoupling, and to augment eNOS activity. Although their precise mode of action remains to be clarified, AVE are likely to interact with eNOS uncoupling. Subsequently, in **chapter 5**, we test if AVE3085 can prevent pressure overload-induced left ventricular (LV) hypertrophy. We have demonstrated that AVE 3085 attenuates the pathological LV changes caused by pressure overload. Surprisingly, these effects are seen in the absence of transcriptional upregulation of eNOS. Importantly, we provide a novel beneficial function of AVE3085 treatment on eNOS stability, demonstrating by an increase in eNOS dimer to monomer ratio and by a decrease in myocardial ROS generation. This effect is likely the result of protection of BH4 against oxidation by ROS. Possible mechanisms for AVE3085 mediate modulation/dampening of transverse aortic constriction-induced inflammatory responses and fibrosis may result from (1) indirect effects secondary to inhibition of macrophage infiltration and inflammatory signaling mediated by NO and/ or BH4, or (2) pleiotropic effects of AVE3085 on anti-inflammatory and anti-fibrotic effect.

The second part of this thesis is centered on the role of LEC in atherosclerosis. Angiogenesis, the sprouting of new blood vessels from the pre-existing ones, is essential for physiological development. Pathological angiogenesis contributes to pathogenesis of cardiovascular diseases. Although plaque angiogenesis has been linked to proinflammatory and proatherosclerotic effects, lymphatic vessels have only recently been implicated in the pathogenesis of MI and atherosclerosis, where proper lymphatic drainage seemed to protect against disease. The lymphatic system is increasingly recognized as a critical process in atherosclerosis to transport cholesterol and immune cells to the lymph nodes and eventually to the circulation. However, the precise driving factors and consequences of lymphangiogenesis in the context of human atherosclerosis still remain to be elucidated. Plaque-resident factors specifically involved in pathologic lymphangiogenesis during atherosclerosis development and progression may well represent novel approaches for therapeutic modulation of plaque lymphangiogenesis. In this part, it is hypothesized that gene network analysis can provide powerful predictions of candidate genes and those genes represent potential novel targets for regulating lymphangiogenesis-associated atherosclerosis in human (approaches will be described below).

In **chapter 6** we attempt to identify novel players in pathologic lymphangiogenesis in atherosclerosis. We employ a bioinformatics approach, weighted gene coexpression network analysis (WGCNA), to identify networks and candidate genes relevant to plaque lymphangiogenesis in humans. In this study, we correlate phenotypic traits i.e. LVD, identified by D2-40⁺ in human atherosclerotic tissue with transcription profiles, derived from the same tissue. We are able to identify modules with high correlation to LVD, unveiling a novel role for the phosphatase TNIP2, as a VEGF- independent key regulator of plaque lymphangiogenesis in humans *ex vivo*, in LEC *in vitro* and in zebrafish *in vivo*. In addition, we also provide transcription factor binding sites, miRNAs, and potential pathways which may be involved in TNIP2-mediated plaque lymphangiogenesis. TNIP2-responsive genes are enriched in lymphatic signature genes and suggest the involvement of type I/II IFN signaling, and lipid/cholesterol homeostatic regulation in TNIP2 functional effects.

Finally, in **chapter 7** we have discussed the most relevant findings of this thesis and provided the future perspectives. Altogether, this thesis affords novel insights into the molecular regulation of endothelial cells in CVD, with particular focus on eNOS and TNIP2. In conclusion, enhancement of functional eNOS can ameliorate endothelial and heart function in heart failure. In vessels, TNIP2 induction could represent an interesting target to stimulate plaque lymphangiogenesis, thereby promoting inflammation resolution in plaque. Considering the therapeutic potential of my findings we may conclude that (I) AVE3085 helps maintaining eNOS functionality and thus may improve eNOS/NO signaling dependent cardiovascular function, and (II) TNIP2 induction will increase lymphangiogenic responses in plaque, which could improve lymphatic drainage of constituents, cytokines and inflammatory cells from plaque ,and thus may dampen atherosclerosis progression. Therefore, the next challenge is to translate these findings to clinical applications for cardiovascular diseases prevention and/or treatment.

Samenvatting

Samenvatting

Hart- en vaatziekten (HVZ) zijn nog altijd de meest voorkomende doodsoorzaak en vertegenwoordigen 31% van alle sterfgevallen in wereldwijd. Belangrijk is dat atherosclerose verreweg de belangrijkste oorzaak is van HVZ, goed voor 80% van alle HVZ wereldwijd. Atherosclerose is een chronische ontstekingsziekte, geïnitieerd door verhoogde lipide bloedspiegels, die leidt tot de vorming van atherosclerotische plaques in de grote slagaders. Deze plaques bestaan uit afzettingen van cholesterol, lipiden en cellulaire resten in de vaatwand, wat resulteert in een vernauwing van de slagaders. Vroege atherosclerotische plaques worden gekenmerkt door subendotheliale accumulatie van macrofagen en macrofaag schuimcellen. In gevorderde stadia van de ziekte zullen mediale gladde spiercellen prolifereren, migreren naar de intima en collageen produceren, waarbij een vezelachtige kap wordt gevormd die de plaque bedekt (d.w.z. een stabiele plaque). De instabiele plaque wordt daarentegen gekenmerkt door een grote, lipidenrijke kern in, veel ontstekingscellen en een dunne fibreuze kap. Als de instabiele plaque scheurt, leidt dit tot thrombusvorming, afsluiting van het lumen, en vervolgens tot distale ischemie en acute cardiovasculaire aandoeningen, zoals een hartinfarct of beroerte.

Endotheelcellen spelen een belangrijke rol bij het handhaven van cardiovasculaire homeostase. In dit proefschrift hebben we ons gericht op de moleculaire regulatoren van het endotheel. Het doel van dit proefschrift is tweeledig: 1) onderzoek naar endotheelcel (EC) dysfunctie gemedieerd door verstoorde functie van stikstofoxide (NO) bij hartfalen, en 2) identificatie van nieuwe moleculaire regulatoren van lymfatische EC (LEC) in atherosclerose.

Het eerste deel van het proefschrift (**hoofdstuk 2-5**) bestudeert endotheel dysfunctie in hart en bloedvaten, wat vaak wordt gekenmerkt door een verminderde biologische beschikbaarheid van NO. Dit veroorzaakt vasoconstrictie, coagulatie en ontsteking, en leidt bij chronische dysfunctie tot verschillende pathologische aandoeningen, waaronder een hartinfarct, hypertensie en atherosclerose. In dit proefschrift wordt de hypothese geformuleerd dat hypertrofie van het linkerventrikel geïnduceerd door drukoverbelasting verminderd kan worden door modulatie van endotheel NO synthase (eNOS).

In **hoofdstuk 2** wordt de rol van tetrahydrobiopterin (BH4) besproken, een belangrijke regulator van eNOS functie en vervolgens van cardiovasculaire homeostase en HVZ. De biologische beschikbaarheid van BH4 kan gehandhaafd worden door 1) het verbeteren van de regeneratie van BH4 uit de inactieve vorm BH2, 2) chemische stabilisatie van BH4 en 3) het verminderen van oxidatieve afbraak van BH4. Inderdaad heeft eNOS-afhankelijke productie van NO een cruciale rol in de regulatie van de endotheel functie. **Hoofdstuk 3** geeft een uitgebreid overzicht van de

belangrijke rol van eNOS-ontkoppeling in de pathogenese van endotheel disfunctie. De stabilisatie van eNOS-functie en het onderdrukken van radikaal (ROS) productie door eNOS zijn veelbelovende therapeutische methoden voor het verbeteren van endotheel disfunctie. Vervolgens worden in **hoofdstuk 4** twee nieuwe farmacologische stoffen besproken, AVE9488 en AVE3085. Deze stoffen zijn ontworpen om eNOS-transcriptie te verhogen, en NO-signalering en biologische beschikbaarheid te verbeteren. Interessant genoeg, verhogen AVE's ook de biologische beschikbaarheid van BH4, wat de ontkoppeling van eNOS omkeert, en zo de eNOS-activiteit versterkt. Hoewel het precieze mechanisme nog moet worden opgehelderd, heeft AVE waarschijnlijk een wisselwerking met de ontkoppeling van eNOS. Vervolgens bestuderen we in **hoofdstuk 5** of AVE3085 linker ventrikel (LV) hypertrofie kan voorkomen. We hebben aangetoond dat AVE 3085 de pathologische veranderingen in het LV die worden veroorzaakt door drukoverbelasting vermindert. Verrassenderwijs worden deze effecten waargenomen in de afwezigheid van verhoogde genexpressie van eNOS. Belangrijk is dat we een nieuw gunstige effect van de behandeling met AVE3085 op de stabiliteit van eNOS hebben ontdekt, wat blijkt uit de toegenomen verhouding van eNOS-dimeer tot monomeer, en verlaagde ROS-generatie. Dit effect is waarschijnlijk het gevolg van de bescherming van BH4 tegen oxidatie door ROS. De AVE3085-afhankelijke vermindering LV ontstekingsreacties en fibrose kunnen het gevolg zijn van (1) indirecte effecten secundair aan de remming van macrofaag infiltratie en ontstekingssignalering gemedieerd door NO en / of BH4, of (2) pleiotrope effecten van AVE3085 op ontsteking- en fibrose remming.

Het tweede deel van dit proefschrift concentreert zich op de rol van LEC bij atherosclerose. Angiogenese, het ontspruiten van nieuwe, kleine bloedvaten uit reeds bestaande bloedvaten, is essentieel voor de fysiologische ontwikkeling. Pathologische angiogenese draagt daarentegen bij aan de ontwikkeling van HVZ. Hoewel plaque angiogenese is gekoppeld aan pro-inflammatoire en pro-atherosclerotische effecten, is het beschermende effect van drainage door lymfevaten in de ontwikkeling van HVS in proefdieren pas recent aangetoond. Het lymfestelsel wordt steeds meer herkend als een essentiële link in atherogenese, om cholesterol en immuun cellen via de lymfeklieren af te voeren naar het bloed. De precieze regulatoren en consequenties van lymfangiogenese in de context van humane atherosclerose moeten echter nog worden opgehelderd. Plaque factoren die specifiek betrokken zijn bij de vorming van pathologische lymfangiogenese tijdens de ontwikkeling en progressie van atherosclerose, zijn mogelijk interessante nieuwe aangrijpingspunten voor de interventie in plaque-lymfangiogenese. In dit deel wordt de hypothese getest dat netwerkanalyse een krachtige methode is kandidaat genen te identificeren die lymfangiogenese in humane atherosclerose reguleren.

In **hoofdstuk 6** gebruiken we een bio-informatica methode, gewogen gencoëxpressie-netwerkanalyse (WGCNA), om netwerken en kandidaat-genen te identificeren die relevant zijn voor plaque-lymfangiogenese bij mensen. In deze studie correleren we fenotypische kenmerken, d.w.z. dichtheid van D2-40+ lymfevaten (LVD) in humane atherosclerotische plaques met genexpressie patronen van dezelfde plaque. We hebben inderdaad gen modules geïdentificeerd die sterk correleerden met LVD. Er is een nieuwe functie vastgesteld voor de fosfatase TNIP2, die – onafhankelijk van VEGF, plaque-lymfangiogenese reguleert bij de mens ex vivo, LEC in vitro en in de zebrafish in vivo. Daarnaast leverde deze bio informatica methode ook transcriptiefactoren, micro RNA's en potentiële cellulaire processen op, die de regulatie van plaque lymfangiogenese door TNIP2 verklaren. TNIP2-afhankelijke genen zijn verrijkt in een genset, waarvan bekend is dat deze genen lymfangiogenese reguleren. Ook zijn TNIP2-afhankelijke genen betrokken bij type I / II IFN-signalering en lipide/cholesterol metabolisme.

Ten slotte worden in **hoofdstuk 7** de meest relevante bevindingen van dit proefschrift en de toekomstperspectieven besproken. Samenvattend, biedt dit proefschrift nieuwe inzichten in de moleculaire regulatie van endotheelcellen bij HVZ, in het bijzonder onder invloed van eNOS en TNIP2. We concluderen ten eerste dat verbetering van functioneel eNOS de functie van endotheelcellen en het hart bij hartfalen kunnen verbeteren. Ten tweeden, kan stimulatie van TNIP2 in slagaders een interessant aanpak vormen voor het stimuleren van plaque-lymfangiogenese, om de ontstekingsresolutie in plaques te verbeteren. Wat betreft de therapeutische mogelijkheden van mijn bevindingen kunnen we concluderen dat (I) AVE3085 helpt de eNOS-functionaliteit te behouden en daardoor de eNOS / NO-afhankelijke cardiovasculaire functie kan verbeteren, en (II) stimulatie van TNIP2 kan de lymfangiogenese reacties in plaque verhogen, wat de lymfedrainage van lipiden, cytokines en ontstekingscellen van plaque kan verbeteren, en zon de progressie van atherosclerose kan remmen. De volgende uitdaging is de om deze bevindingen te vertalen naar de patiënt voor preventie en / of behandeling van hart- en vaatziekten.

Valorization

Valorization

Social and economic value of the current thesis

Cardiovascular disease (CVD) is the most costly class of diseases, in comparison to other major diagnostic groups. Thus it accounts for an immense global health and economic burden ¹. A staggering 17 million people worldwide die annually from myocardial infarction and stroke (www.who.org). In the United States, CVD was responsible for an estimated health expenditure of around \$316.1 billion in 2012 and 2013 ¹, and projections are even more alarming, with total costs expected to increase by 117 % between 2012 and 2030 ¹. Atherosclerosis, specifically the rupture of an atherosclerotic plaque, is the main underlying cause of this tremendous cardiovascular mortality and morbidity ². Despite pharmacological advancements, no therapy is presently able to fully eradicate atherosclerosis or prevent plaque rupture ³.

The studies contained within this thesis investigated novel therapeutic options in CVD, focusing on (I) heart failure as a consequence of pressure overload, and (II) atherosclerosis. Subsequently, we have demonstrated that (I) the small molecule AVE3085 helps to maintain eNOS function, and thus may improve eNOS/NO signaling-dependent cardiovascular function, and (II) TNIP2 induction is associated with lymphangiogenic responses in the atherosclerotic plaque, while TNIP2 knockdown prevented normal lymphangiogenic development *in vitro* and in zebrafish *in vivo*. This secondary finding implies that TNIP2 induction could improve lymphatic drainage of constituents, cytokines and inflammatory cells from human plaques, thus potentially dampening atherosclerosis progression. These findings are relevant both to healthcare providers and to individuals at risk for CVD. Furthermore, the knowledge acquired here will directly contribute to the expertise of the cardiovascular researchers involved, allowing them to continue to advance our understanding of the molecular mechanisms of CVD. While this thesis presents data from basic research, and is not applicable for direct translation into clinical practice, it nevertheless provides a novel starting point for future developments in cardiovascular research, and potential for the development of new therapies.

Current and future treatment of heart failure

Classification of heart failure (HF) is based on left ventricular ejection fraction (EF). A reduced EF in patients with clinical signs and symptoms of HF is referred to as, HF with reduced ejection fraction (HFrEF). However, more than 50 % of all HF patients exhibit HF with preserved ejection fraction (HFpEF). These patients are predominantly elderly women, and have high rates of associated comorbidities, such as obesity and hypertension ^{4, 5}. Unlike HFrEF, the diagnosis of HFpEF is difficult; being easily missed by echocardiography - especially in patients who often show

normal EF, have multiple comorbidities and have no obvious physical signs of fluid overload^{6, 7}.

HF cannot be cured, but it can be treated. The goal of treatment in patients with HF is to improve their clinical status, functional capacity and quality of life, preventing hospital admission and reducing mortality^{8, 9}. However, life prolongation depends upon the inhibition of cardiac remodeling¹⁰. Anti-neuroendocrine treatment (i.e. angiotensin converting enzyme inhibitors, mineralocorticoid receptor antagonists, and beta-blockers) has been shown to reverse modeling, and is proven to be effective in treating patients with HFrEF. In contrast, modern heart failure pharmacotherapy has not been shown to improve outcome in HFpEF^{4, 9, 11}. As such, there is a great unmet need for new therapeutic approaches for HFpEF. It has been well documented that oxidative stress, decreased NO bioavailability and dysfunctional eNOS (uncoupled eNOS) contribute to myocardial remodeling and dysfunction in HFrEF and HFpEF^{12, 13}. This indicates that maintaining or restoring eNOS functionality, subsequently increasing NO bioavailability, is a promising therapeutic intervention in HF.

Perspectives on AVE3085

In chapter 5, we have demonstrated that AVE 3085 attenuates the pathological left ventricular changes caused by pressure overload. AVE 3085 enhanced eNOS function by restoring its coupling ability, and subsequently reducing myocardial reactive oxygen species (ROS) generation. This effect most likely results from increased protection of BH4 from ROS-mediated oxidation. In addition, AVE3085 has been shown to ameliorate diastolic dysfunction and reverse cardiac remodeling in DAHL; a salt sensitive rat model of HFpEF¹⁴. These findings create new opportunities to use the eNOS/ NO pathway as a therapeutic target in the treatment of HF.

AVE3085 may have advantages over several other eNOS modulators. For instance, administration of BH4 has been shown to improve systolic and diastolic function in experimental HF^{15, 16}. However, BH4 has a relatively narrow dose–response window, which remains a point of concern for the translatability of BH4 supplementation for clinical disease^{17, 18}. Upon oral administration, BH4 is largely oxidized to BH2, which can then be re-reduced once inside the cell. This conversion may be limited by diseases with oxidative stress¹⁹. Moreover, higher doses of BH4 might tip the balance toward more BH2, which can then compete with BH4 to impair eNOS coupling, leading to a paradoxical reversal of benefit^{17, 18}. This suggests that alternative pharmacological approaches to prevent BH4 oxidation or increase BH4 biosynthesis may be a more rational therapeutic strategy to improve eNOS functionality²⁰. This can potentially be accomplished by AVE3085 alone, or in combination with other eNOS modulators; restoring BH4 bioavailability to

therapeutically perturb nitroso-redox balance in cardiovascular disease. For instance, folic acid and its metabolically active form 5-methyltetrahydrofolate (5-MTHF), increased binding affinity of BH4 to eNOS, and enhanced the regeneration of BH4 from its inactive form dihydrobiopterin (BH2)^{21, 22}. Moreover, Vitamin C or L-ascorbic acid have been shown to stabilize BH4²³. Clearly, additional studies are needed to further clarify the potential role of AVE3085, with or without co-supplementation, for the treatment and/or prevention of cardiovascular disease. In future studies, I would determine the effect of AVE3085 in eNOS-deficient mice to confirm that AVE3085 is indeed eNOS-dependent. Furthermore, I would evaluate the effect of AVE3085 on reversal of pre-existing pressure overload-induced compensated cardiac dilation and decompensated dilation mice models, as this pathophysiology is more relevant to the patient clinical situation. In fact, AVE3085 has entered Phase I clinical trials for congestive heart failure in Europe (Sanofi Aventis business report 2007) and thus, has undergone pharmacokinetic and pharmacodynamic studies. The future study could therefore start at Phase II: according to the timeline of drug developments²⁴, if AVE3085 is approved by FDA, it may be launched to the market in a minimum of 7-10 years.

Current and future treatment of atherosclerosis

Atherosclerosis is a chronic inflammatory disease associated with dyslipidemia²⁵. In addition to healthy lifestyle changes, current atherosclerosis therapies focus on lowering blood cholesterol levels, improving blood pressure control and preventing thrombotic complications²⁶. However, these strategies are not effective in all patients, and do not directly address the inflammatory mechanisms driving atheroprogession^{27, 28}. Results from the recent clinical trial “CANTOS” suggested that targeted anti-inflammatory therapy, i.e. canakinumab, a therapeutic monoclonal antibody targeting interleukin-1 β , leads to reduced cardiovascular risk and atherosclerosis, without lowering cholesterol level²⁹. However, effect on cardiovascular mortality was not observed in this trial, and patients who received canakinumab were at greater risk of death from infection than those who received a placebo³⁰. The tremendous costs associated with this new compound, along with minimal health benefits, indicate that new therapeutic interventions able to decrease metabolic disorder-associated inflammation are required. Emerging evidence has strongly suggested that lymphatics are critical for immune response and cholesterol metabolism in atherosclerosis, indicating an essential contribution of lymphangiogenesis to atherosclerosis regression. Below, I will discuss prolymphangiogenesis as a potential novel therapeutic approach for atherosclerosis.

Therapeutic lymphangiogenesis – a new avenue for atherosclerosis treatment

The role of angiogenesis in atherosclerosis has been studied extensively; however, research on plaque lymphangiogenesis is relatively new. Lymphatics can be detected in human as well as mouse atherosclerotic plaque adventitia³¹⁻³³, suggesting a contribution to atherosclerotic lesion progression. Enhancement of lymphatic function represents a potential therapeutic target in atherosclerosis, through the promotion of immune cell (e.g. macrophage) egression from the plaque, and prevention of cholesterol accumulation. In addition, prolymphangiogenic therapy may prevent local edema, caused by leakage from the plaque vasa vasorum³², or enhance the efficiency of proangiogenic therapy in CVD, by reducing revascularization and angiogenesis-related edema³⁴. Advances in understanding the molecular underpinning of plaque lymphangiogenesis will offer insight into therapeutic options, potentially leading to the discovery of novel lymphangiogenic agents able to reverse atherosclerosis.

The identification of suitable target genes to influence the disease development is necessary for successful gene therapy²⁸. In **chapter 6**, we provided a new insight in bioinformatics; demonstrating the success of a weighted gene coexpression network analysis (WGCNA) in identifying and validating key regulators of plaque lymphangiogenesis. Unlike a more traditional approach that focuses on differentially expressed individual genes, our approach is unique: we have employed WGCNA to capture high-order gene–gene interrelation in association with phenotypic trait i.e. plaque lymphangiogenesis. WGCNA gives information about functional connections, providing a true systems medicine perspective³⁵. To provide proof of concept that WGCNA can identify functionally relevant genes, in **chapter 6**, we have investigated the role of the candidate genes, and demonstrated that TNIP2 is indeed a novel regulator of inflammatory lymphangiogenesis. Together, we provide insights that WGCNA can effectively integrate cardiovascular gene expression and trait data, to identify novel therapeutic targets or potential genetic biomarkers of the diseases.

Perspectives on TNIP2 as a novel regulator of inflammatory lymphangiogenesis

We show in **chapter 6** that TNIP2 appears to be a specific regulator of pathologic lymphangiogenesis in atherosclerotic plaque instability. We postulate that TNIP2 may represent a much more suitable clinical target, possibly superior to other prolymphangiogenic agents. For instance, VEGF-C treatment has been reported to induce angiogenesis, increase inflammation and induce blood vascular leakage, leading to local edema which further impairs lymphatic function in experimental animal models^{36,37}. These deleterious effects may promote plaque instability and rupture. In contrast, TNIP2 is not a growth factor per se, but interacts with A20, which exerts anti-

atherogenic properties^{38,39}. TNIP2 did not affect angiogenesis in our zebrafish studies and possibly would not promote the blood vascular “side effects”. Nevertheless, the role of TNIP2 in angiogenesis remains to be elucidated. The beneficial effects of TNIP2 may extend beyond its prolymphangiogenic properties, as TNIP2 can interfere with the inflammatory process in atherogenesis at multiple levels, i.e. NFκB, the Ang1/Tie2 axis, PPARs and IFNγ. It is well known that NFκB directly controls several proatherogenic genes and thus integrates multiple processes contributing to the formation of atherosclerotic plaques⁴⁰. Indeed, TNIP2 exerts inhibitory effects on NFκB activation⁴¹. Moreover, it has been suggested that Ang 1/Tie2 exhibits anti-atherogenic effects⁴². Interestingly, TNIP2 structurally and functionally interacts with Ang1/ Tie2 signaling, leading to the inhibition of NFκB –dependent inflammatory gene expression⁴³⁻⁴⁵. Importantly, it has been suggested that anti-inflammatory drugs with pro-lymphangiogenic activity are appealing treat inflammatory conditions. This is because the activation of lymphatic function reduces the severity of tissue inflammation and thus, contributes to accelerated inflammation resolution^{46, 47}. **In chapter 6**, we have highlighted such a regulatory role for TNIP2 in the PROX1 axis, enhancing PPAR-related lipid and cholesterol metabolism signaling and inhibiting that of IFNγ; indicating that TNIP2 exerts, not only lymphangiogenic, but also anti-inflammatory effects in lymphatic endothelial cells. Together, these findings suggest that TNIP2 may be an interesting target for the development of novel therapeutic strategies to treat inflammatory diseases, particularly in atherosclerosis. Further steps will have to be taken to confirm the findings in relevant animal models of atherosclerosis. For instance, target validation studies can be tested by overexpression or deletion of TNIP2 in atherogenic ApoE-deficient mice, or in mice with perivascular carotid collar placement-induced atherosclerosis. Subsequently, a small molecular weight chemical compound (SMOL) should be designed and tested *in vitro* and *in vivo*. In addition, the protection of intellectual property is required for the potential compounds, in order to eliminate the competition and to gain the freedom to operate⁴⁸. Furthermore, large animal efficacy and toxicity studies are required; for which I prefer PCSK9-overexpressing mini pigs^{49,50}, as their phenotype more closely resembles human plaques. Importantly, porcine models allow investigation of the impact of adventitial neovascularization by coronary imaging technologies^{51,52}. Thus, this may suitable to investigate adventitial lymphangiogenesis, and therefore will be easier to translate the results to the human situation. Subsequently, the suitable drug will eventually be utilized in multiple phases of human clinical trials and human randomized control studies. The particular target patients include those afflicted with atherosclerosis, MI and potentially lymphedema. In total, the *de novo* drug development requires 10-20 years before market launch²⁴. GlaxoSmithKline may here be involved in developing the compounds; the company has an ongoing investigation of the TNIP2/ TPL-2/ NFκB1 p105 complex for anti-inflammatory pharmaceuticals⁵³.

In conclusion, although our findings cannot directly be translated into clinical application, we have provided a very promising concept which has therapeutic potential in future treatment and prevention of CVD. Further studies in combination with experimental verification are strongly encouraged to clarify their detailed role, and subsequently meet their clinical reality.

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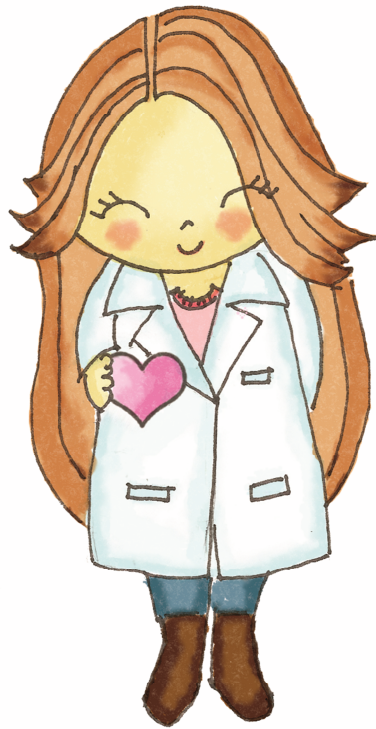
List of abbreviations

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5-MTHF	5-methyltetrahydrofolate
ACE	angiotensin-converting enzyme
ACS	acute coronary syndromes
ACVRL1	activin A receptor like type 1
ADMA	asymmetric dimethylarginine
Ang	angiopoetin
ANP	A-type natriuretic peptide
apoE	apolipoprotein E
AT2	angiotensin II
b.i.d.	bis in die/ two times a day
BEC	blood endothelial cells
BH2	dihydrobiopterin
BH4	tetrahydrobiopterin
BMC	bone marrow-derived mononuclear cells
BMI	body mass index
BNP	B-type natriuretic peptide
cav-1	caveolin-1
CHD	coronary heart disease
CHF	congestive heart failure
COUP-TFII	chicken ovalbumin upstream promoter-transcription factor II
CRP	C-reactive protein
CVD	cardiovascular disease
DAB	3,3'-Diaminobenzidine
DHF	diastolic heart failure
DHFR	dihydrofolate reductase
DHPR	dihydropteridine reductase
DLL4	delta-like 4
DM	diabetes mellitus
dpf	days post-fertilization
EC	endothelial cells
EDHF	endothelium-derived hyperpolarization factor
EDR	endothelium-dependent relaxation
EF	ejection fraction
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cells
FA	folic acid
Fe ⁺⁺	ferrous ion
FGF	fibroblast growth factor

Foxc2	forkhead box C2
FS	fraction shortening
GO	gene ontology
GTPCH	GTP cyclohydrolase
HDL	high-density lipoprotein
HE	hematoxylin and eosin
HF	heart failure
HFmrEF	heart failure with mid-range ejection fraction
HFpEF	heart failure with preserved ejection fraction
HFrfEF	heart failure with reduced ejection fraction
HIF	hypoxia-inducible factor
hpf	hours post-fertilization
HUVEC	human umbilical vein endothelial cells
I/R	ischemia/reperfusion
ICAM-1	intracellular adhesion molecule-1
ICMP	ischemic cardiomyopathy
IFNG/ γ	interferon gamma
IHD	ischemic heart disease
IL1B/ β	interleukin 1 beta
IPH	intraplaque haemorrhage
ISVs	intersegmental vessels
LEC	lymphatic endothelial cells
LPS	lipopolysaccharide
LV	left ventricular
LVD	lymphatic vessel density
LVEDD	left ventricular end-diastolic diameter
LVEDS	left ventricular end-systolic diameter
LYVE-1	lymphatic vessel hyaluronan receptor 1
MDA	malondialdehyde
MHC	myosin heavy chain
MI	myocardial infarction
miR	microRNA
mtNOS	mitochondrial NOS
MVD	microvessel density
NFkB	nuclear factor kappa beta
NO	nitric oxide
Notch1	notch homolog 1, translocation associated (Drosophila)
NOX	NADPH oxidases
NRP2	neuropilin-2
ORA	over-representation analysis
oxLDL	oxidized-low density lipoprotein

p.o.	per os/ orally
PAD	peripheral arterial disease
PDPN	podoplanin
PKG	protein kinase G
PLB	phospholamban
PLs	parachordal vessels
PPARG/ γ	peroxisome proliferator-activated receptor gamma
PPIN	protein-protein interaction network
PROX1	prospero homeobox 1
PSR	picro-sirius red
PSS	plaque structural stress
RCT	reverse cholesterol transport
RIN	RNA Integrity Number
ROS	reactive oxygen species
SDF-1	stromal cell-derived factor 1
siRNA	small interfering RNA
SMC	smooth muscle cells
SOX18	sex determining region Y box 18
STAT1	signal transducer and activator of transcription 1
STP	staurosporine
STZ	streptozotocin
TAC	transverse aortic constriction
TD	thoracic duct
TFBS	transcription factor binding site
TFCA	thin-cap fibroatheromas
TGF- β	transforming growth factor beta
TLR4	toll-like receptor 4
TNFA/ α	tumor necrosis factor alpha
TNIP2	TNFAIP3 Interacting Protein 2
VASP	vasodilator-stimulated phosphoprotein
VCAM-1	vascular cell adhesion molecule-1
VEC	venous endothelial cells
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VSMC	vascular smooth muscle cells
VV	vasa vasorum
WGCNA	weighted gene co-expression network analysis
WSS	wall (or endothelial) shear stress
WT	wild-type
XO	xanthine oxidase



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Dankwoord

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PUBLICATIONS

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MEETING ABSTRACTS

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